Perinatal bisphenol A exposure: Effects on metabolic homeostasis mediated by epigenetic labile loci

By

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ABSTRACT

Epidemiological and animal studies firmly establish that nutritional and environmental exposures during early embryonic development play a critical role in adult disease susceptibility, such as obesity, type 2 diabetes, and neurodevelopmental disorders. Epigenetic reprogramming is a molecular consequence of exposures during critical developmental periods. Linking environmental exposures to adult disease via epigenetic mechanisms may help our understanding of human complex disease prevention, diagnosis, and treatment.

Bisphenol A (BPA), a high production-volume chemical monomer used in polycarbonate plastic and epoxy resins, is found in consumer products including food and water containers, receipts, baby bottles, and metal can linings. Due to its ubiquitous presence in the environment and ability to leach from consumer products, humans are continuously exposed to BPA. Thus, several studies have measured detectable levels of BPA in urine, breast milk, and placental tissue in populations around the world including the United States, Korea, and China.

This dissertation develops and utilizes a dose-dependent mouse model of multiple human physiologically relevant levels of BPA exposure (50 ng, 50 ∓g, and 50 mg BPA/kg diet) *in utero* and during lactation, and evaluates BPA's influence on offspring metabolic parameters throughout the life-course, hormonal status at 9 and 10 months, and epigenetic gene regulation at post-natal day 22 and 10 months. First, perinatal BPA exposure was associated with several metabolic phenotypes at 3, 6, and 9 months of age, including increased spontaneous activity, increased energy expenditure, and decreased body weight and fat in offspring. These effects

were more prominent in female, rather than male offspring. Additionally, females at the highest exposure dose displayed an increase in insulin sensitivity, increased adiponectin levels, and decreased leptin levels. Thus, life-course analysis illustrates that perinatal BPA exposure is associated with hyperactive and lean phenotypes.

Second, developmental BPA exposure was linked to alterations to the epigenome in early life (postnatal day 22), including global DNA methylation levels as well as candidate gene methylation levels at two well-characterized metastable epialleles and five additional genes linked to metabolic homeostasis. Specifically, global DNA methylation among CCGG site throughout the genome were significantly decreased in all BPA exposure groups. Offspring exposed to the highest BPA exposure dose displayed hypomethylation at the A^{vy} locus. Offspring exposed to the middle BPA exposure dose displayed hyper- and hypomethylation at $Cabp^{IAP}$ and *H19*, respectively. Finally, offspring exposed to the high and low exposure doses displayed hypermethylation at *Igf2*. These epigenetic data suggest that developmental BPA exposure through the maternal diet is altering normal levels as compared to controls of DNA methylation in candidate genes, as well as globally in PND 22 tissue, and may program individuals for later in life disease susceptibility.

Lastly, an epigenome-wide discovery platform was utilized to elucidate epigenetic alterations in adult offspring (10 months of age) following perinatal BPA exposure. Biological pathway analysis was run on the significant differentially methylated regions among females to determine enriched pathways that may be involved in the metabolic or hyperactive phenotypes displayed in the BPA exposed offspring from the metabolic phenotyping. Furthermore, utilizing the top enriched biological pathways, three candidate genes were chosen to assess DNA methylation as a mediating factor linking the association of perinatal BPA exposure to the

metabolic life-course analysis. DNA methylation in two top hit candidate genes, *Irs-2* and *Jak-2*, was linked as a potential mediator in the mechanistic pathways in BPA exposed females and the adiponectin and glucose phenotypes, respectively.

In summary, this project illustrates that perinatal BPA exposure is associated with hyperactive and lean murine phenotypes mediated by alterations to normal DNA methylation patterns. Data generated from this study is crucial for deciphering the role of epigenetics in the pathogenesis of chronic disease and the development of novel epigenetic-based prevention and therapeutic strategies for complex human disease.

CHAPTER 1

Introduction

1.1 Overview of Dissertation

The overall theme of this dissertation is the role of early life chemical and nutritional exposures on the susceptibility of disease formation in adulthood. A growing body of work supports the developmental origins of health and disease (DOHaD) hypothesis, in which environmental insults early in development influence chronic disease outcomes such as obesity, type 2 diabetes, cancer, and cardiovascular disease later in life [Barker, 2004; Bateson et al., 2004]. The mechanistic link between what individuals are exposed to in early life and disease formation throughout the life-course appears to involve alterations to epigenetic modifications such as DNA methylation at labile loci. Epigenetics is the study of mitotically heritable yet potentially reversible, molecular modifications to DNA and chromatin without alteration to the underlying DNA sequence [Li, 2002; Reik et al., 2001]. Though DNA sequence is fairly permanent, epigenetic modifications are dynamic throughout the life-course and can be heavily influenced by external factors [Reik et al., 2001]. Thus, external effects on the epigenome may alter gene expression, potentially giving rise to phenotypic disparity including disease formation.

The objective of this dissertation project is to further understand the role of perinatal (gestational and lactational) exposure to multiple physiologically relevant levels of the high production chemical bisphenol A (BPA) on the development of metabolic disease throughout the life-course and to discover potential epigenetic mechanisms that are influenced by

developmental BPA exposure. Furthermore, it is of importance to strengthen our understanding of the biological pathways associated with developmental insults and adult disease formation by elucidating whether epigenetic mechanisms are mediating these relationships.

1.2 Background

1.2.1 Bisphenol A

Bisphenol A (BPA) is a monomer produced in high volumes and comprises polycarbonate plastics and epoxy resins. It is found in consumer products such as food and beverage containers, baby bottles, dental composites, thermal paper, and the inside lining of metal cans. BPA can leach from these consumer products under high temperature, acidic or basic conditions, presenting many opportunities for human exposure, most commonly via ingestion, and also to a lesser extent via inhalation and dermal routes [Vandenberg et al., 2010]. Thus, BPA is of health concern because it is ubiquitous in the environment resulting in widespread human exposure. Several studies have reported detectable levels of total urinary BPA in a majority of individuals in populations around the world, including the United States, China, Japan, and Korea [Calafat et al., 2008; Zhang et al., 2011].

BPA can mimic or antagonize endogenous hormones, and subsequently perturb endocrine function [Gould et al., 1998; Kuiper et al., 1998]. BPA has been most widely studied as an environmental estrogen and has binding affinities for estrogen receptors alpha and beta (ER α and β), G protein-coupled receptor 30 (GPR30), and estrogen related receptor gamma (ERR γ) [Thomas et al., 2006]. Dependent upon the dose, cell type, and ER isoform, BPA can exert agonist or antagonist estrogenic activity [Wetherill et al., 2007]. BPA has also been observed to

interfere with thyroid and androgen receptor activity [Kruger et al., 2008; Moriyama et al., 2002]. BPA can activate transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and the aryl hydrocarbon receptor (AhR) [Kruger et al., 2008; Sui et al., 2012]. Because these receptor pathways play an important role in gene regulation, BPA may influence normal differentiation and maturation processes during embryonic and fetal development, predisposing individuals to chronic disease development such as obesity, type 2 diabetes, and metabolic syndrome throughout the life-course [Bateson et al., 2004].

1.2.2 Bisphenol A, Obesity, and Metabolic Disorders

The dramatic rise in obesity over the last few decades has led scientists to look beyond excessive calorie intake and lack of physical activity as the main contributing factors [Holtcamp, 2012]. Concurrently, over the past several decades developmental environmental insults, including BPA exposure, have exhibited the ability to perturb the metabolic signaling that play key roles in adipose tissue biology and the homeostatic control of appetite and satiety [MacKay et al., 2013]. Consequently, these environmental insults resulting in long lasting obesity phenotypes have been labeled as obesogens [Blumberg et al., 2011; Grün et al., 2006].

Increasingly animal studies indicate that BPA can disrupt metabolic homeostasis by interfering with endocrine hormone pathways such as thyroid, estrogen, and androgen receptors resulting in a variety of metabolic disorders [Alonso-Magdalena et al., 2008; Moriyama et al., 2002; Rubin et al., 2009]. For example, BPA administered subcutaneously in 8 week old male mice was associated with hyperinsulinemia without a change in blood glucose demonstrating a classic indication of insulin resistance [Alonso-Magdalena et al., 2006]. Furthermore, the female offspring of rats fed a BPA supplemented diet during gestation exhibited glucose intolerance and

decreased insulin sensitivity at 6 months of age, both signs of type 2 diabetes [Alonso-Magdalena et al., 2010]. Perinatal BPA exposure has also been associated with overexpression of lipogenic genes such as CAAT enhancer binding protein alpha, peroxisome proliferator-activated receptor gamma (PPAR-γ), and lipoprotein lipase leading to enhanced adipogenesis [Somm et al., 2009]. Peri- and postnatal BPA exposure has resulted in an increase in body weight due to increased adipose tissue mass accompanied by increased total cholesterol and triacylglycerol [Miyawaki et al., 2007; Somm et al., 2009]. PPAR- γ is a key regulator in adipose cell differentiation [Tontonoz et al., 2008]. *In vitro* studies reveal enhanced expression of PPAR- γ in 3T3-L1 pre-adipocytes promoting increased production of adipocytes [Sargis et al., 2010]. In human subcutaneous and visceral adipose tissue explants, BPA not only inhibited the release of adiponectin but also stimulated the release of inflammatory adipokines interleukin-6 and tumor necrosis factor α, common inflammatory markers found in overweight or obese individuals [Ben-Jonathan et al., 2009; Hugo et al., 2008].

Alternatively, there have been reports on developmental BPA exposure and metabolic endpoints that exhibit null results or outcomes that produce weight loss. For example, rats exposed to 2.5 or 5 µg BPA/kg body weight per day from GD 6 through PND 21 had significantly lower body weights compared to controls from PNDs 0 to 21 [Ferguson et al., 2011]. Also, using repeated body weight measurements, CD-1 mice exposed to dietary BPA during gestation and lactation were heavier than controls at 4 weeks of age, but this association was diminished in adulthood, and the effect did not persist in offspring fed a high fat diet [Ryan et al., 2010].

Although human epidemiological studies have shown associations among BPA exposure and metabolic disease in adults, these studies have been limited to cross-sectional design. For

example, in children aged 8-11 years of age, obesity was positively associated with increasing urinary levels of BPA [Wang et al., 2012]. In adults 40 years of age or older, increasing levels of urinary BPA were associated with abdominal obesity and insulin resistance [Wang et al., 2012]. A positive association was found between BPA urinary levels and type 2 diabetes and cardiovascular disease in adult participants of the 2003-04 NHANES [Lang et al., 2008] as well as with coronary heart disease in adult participants of the 2005-06 NHANES [Melzer et al., 2010]. One epidemiological study of longitudinal design demonstrated that urinary BPA levels at 5 years of age was positively associated with body mass index (BMI) at 9 years of age in boys, while prenatal BPA exposure was positively associated with BMI in girls 9 years of age. [Harley, 2013]. Studies of this nature are time-consuming and require long standing cohorts, thus, well-established cohorts are lacking that measure BPA prenatally and follow the children throughout the life-span.

1.2.3 Bisphenol A and Hyperactivity

Despite the increasing evidence describing BPA as an obesogen, another proposed target of BPA involves impairment of central nervous system development [Masuo et al., 2011]. The prevalence of neurodevelopmental disorders like Attention-Deficit and Hyperactivity Disorder (ADHD) and Autism Spectrum Disorder has consistently increased over the past few decades. Although the casual factors involved in the etiology of these disorders is not well understood, environmental exposures like BPA are being explored as contributing factors because of their ability to interfere with neuroendocrine systems during early development [de Cock et al., 2012; Wolstenholme et al., 2011].

Animal studies of perinatal BPA exposure have shown outcomes that include increased spontaneous activity, aggression, anxiety, and altered cognitive function [Braun et al., 2011; Kundakovic et al., 2011]. For example, male mice that were exposed to 0.32 and 0.48 mg/ml BPA at PND 10 exhibited hyperactivity at 2 and 5 months of age [Viberg et al., 2011]. Additionally, male rats exposed to 87 nM BPA at PND 5 displayed hyperactivity compared to their control counterparts measured at 4-5 weeks of age [Ishido et al., 2011]. Finally, perinatal exposure to 2 µg/kg body weight of BPA in male rats was associated with hyperactivity and behavior linked with decreased attention [Zhou et al., 2011]. These animal studies that have demonstrated early developmental BPA exposure resulting in behavioral outcomes have been correlated to human clinical disorders such as ADHD and aggression [Ishido et al., 2004; Matsuda et al., 2010]. Animal models extended to testing both sexes are needed given the evidence that humans maternal BPA exposure is associated with aggression and increased activity among girls [Braun et al., 2009; Zhou et al., 2011].

The mechanistic action on BPA and resulting hyperactive phenotypes is still under investigation, but studies have pointed to the effects of BPA on the dopamergenic system. In male mice exposed perinatally to 2 mg BPA/g of food showed a decrease in dopamine D3 receptor density in the limbic forebrain [Mizuo et al., 2004]. The volume and density of tyrosine hydroxylase neurons, an important rate limiting enzyme in dopamine synthesis, was decreased in the substantia nigra after perinatal exposure to 3 µg BPA/g of food when measured at 11 weeks of age [Tando et al., 2007]. Lastly in male mice exposed to 20 µg/kg BPA perinatally, dopamine levels increased in certain brain regions [Nakamura et al., 2010]. The majority of these mechanistic studies have been limited to male animals, yet the behavioral outcomes are sexually distinct.

1.2.4 Bisphenol A and Sexually Dimorphic Traits

As emphasized above, it is important to explore these hyperactive and other behavioral phenotypes and mechanisms in both genders. During early development BPA can affect the development of sexually dimorphic areas of the brain [Wolstenholme et al., 2011]. For example, in rats exposed to BPA on PND 2, males displayed a significant increase of tyrosine hydroxylase containing neurons in the anteroventral periventricular nucleus of the hypothalamus resulting in neuron levels comparable to females [Patisaul et al., 2006]. Normally females have more tyrosine hydroxylase cells than males. Sex differences in certain brain regions emerge during late gestation and the early post-natal period in rodents and depend on the estrogen and androgen availability. Since BPA can interfere with estrogen and androgen receptors, it has the potential to interfere with the development of these regions resulting in sex-specific health outcomes [Patisaul et al., 2008].

1.2.5 Epigenetics as a Mechanism Linking Bisphenol A Exposure and Health

The developmental origins of health and disease (DOHaD) hypothesis postulates that chemical and/or nutritional exposures during prenatal and early postnatal development influence developmental plasticity, thereby altering susceptibility to adult chronic diseases. These long-term adverse health outcomes due to environmental exposures during development can alter gene expression and affect phenotype by modifying the epigenome [Waterland et al., 2004]. Epigenetics literally means “above the genome,” and comprises the heritable changes in gene expression that occur in the absence of changes to the DNA sequence itself, including chromatin folding and attachment to the nuclear matrix, packaging of DNA around nucleosomes, covalent

modifications of histone tails, and DNA methylation. Epigenetic marks are generally stable in somatic cells, however, during gametogenesis and early pre-implantation the epigenome undergoes extensive reprogramming with the purpose of establishing cell- and tissue-specific gene expression [Hajkova et al., 2002; Reik et al., 2001]. Metastable epialleles are of particular interest during epigenetic reprogramming due to their increased lability to environmental exposures. Once epigenetic marks are established, metastable epialleles, as well as imprinted genes, are generally very stable throughout the life-course. [Dolinoy et al., 2007 ; Waterland et al., 2006; Waterland et al., 2003; Waterland et al., 2006].

Of these epigenetic modifications, DNA methylation is the best studied and characterized. Methylation of DNA occurs when DNA methyltransferases (DNMTs) covalently attach methyl groups from *S*-adenosyl methionine to the carbon-5 position of cytosine bases, generating 5-methylcytosine. In mammals, DNA methylation is primarily a stable repressive mark found in CpG dinucleotides, however its regulation is more dynamic than previously believed [Maunakea et al., 2010]. For example, the epigenome is particularly dynamic during embryogenesis because of extensive DNA synthesis, and the elaborate DNA methylation patterning required for normal tissue development is established during early development, thus making methylation especially susceptible to environmental insults such as BPA [Faulk et al., 2011]

The effects on BPA exposure and DNA methylation have been mostly limited to candidate gene approaches in rodent models. For example, gestational exposure to 5 mg/kg BPA in mice was associated with hypomethylation in CpG sites in the *Hoxa10* promoter region in female offspring [Bromer et al., 2010]. In rats, hypomethylation was observed at the nucleosome binding protein-1 gene promoters and hypermethylation was observed at hippocalcin-like 1 gene

promoter following early neonatal exposure to 10 µg BPA/kg BW/day [Tang et al., 2012]. Additionally, exposure to BPA at 50 mg/kg diet during gestation and lactation has been shown to alter methylation levels at the metastable epialleles A^{vy} and $Cabp^{IAP}$ [Dolinoy et al., 2007]. A limited number of studies have tried to link epigenetic modifications as the mechanism mediating BPA exposure and its association with disease phenotype. One study explored exposure to 10 µg BPA/kg BW/day in male rats during the neonatal period. This exposure resulted in hypomethylation at the *phosphodiesterase type 4 variant 4* gene in prostate cancer cells and was associated with pre-neoplastic prostatic lesions during adulthood implicating that BPA has role in prostate cancer development through altering DNA methylation status in prostate cells [Ho et al., 2006].

This dissertation covers the spectrum of the DOHaD hypothesis by linking the associations of developmental environmental insult (BPA exposure) and resulting adult phenotypes (metabolic phenotypes) to a molecular mechanistic mediator (DNA methylation). First, perinatal BPA exposure was associated with several metabolic phenotypes and hormone levels in adult age. These effects were more prominent in female, rather than male offspring. Second, developmental BPA exposure was linked to alterations in both global and candidate gene DNA methylation levels at 3 weeks of age (day 22). Third, using an epigenome-wide approach, perinatal BPA exposure was associated with altered DNA methylation patterns in liver DNA tissue at 10 months of age. Finally, meditational regression analysis suggests that DNA methylation is a mediator in the mechanistic pathway of BPA exposure and later in life phenotypes.

1.3 Study Design Overview

1.3.1 Mouse Model

This dissertation develops and utilizes a mouse model of early life (gestation and lactation), multiple-dose BPA exposure through the maternal diet. The dietary exposure is designed to alleviate maternal stress as well as mimic continual human BPA exposure, and the timing is designed to capture key DNA methylation reprogramming events occurring at early implantation [Reik et al., 2001]. Mice were generated from a colony that has been maintained with sibling mating and forced heterozygosity for the viable yellow agouti (A^{vy}) allele for over 220 generations, resulting in a genetically invariant background [Waterland et al., 2003]. Animals used in this study were maintained in accordance with the Institute of Laboratory Animal Resources guidelines and were treated humanely and with regard for alleviation of suffering. The experimental protocol was approved by the University of Michigan Committee on Use and Care of Animals.

1.3.2 Dietary Exposure to Bisphenol A and Mating Scheme

Virgin wild-type *a/a* dams, 6 weeks of age, were randomly assigned to one of four phytoestrogen-free AIN-93G diets (diet 95092 with 7% corn oil substituted for 7% soybean oil; Harlan Teklad, Madison, WI): 1) standard diet (n = 11 litters); 2) standard diet supplemented with 50 ng BPA/kg diet (n = 14 litters); 3) standard diet supplemented with 50 µg BPA/kg diet (n = 9 litters); 4) standard diet supplemented with 50 mg BPA/kg diet (n = 13 litters). All diet ingredients were supplied by Harlan Teklad except BPA, which was supplied by NTP (National Toxicology Program, Durham NC). The mg dosage is an order of magnitude lower than the dietary administered maximum non-toxic threshold in rodents (200 mg/kg BW/day) [Takahashi

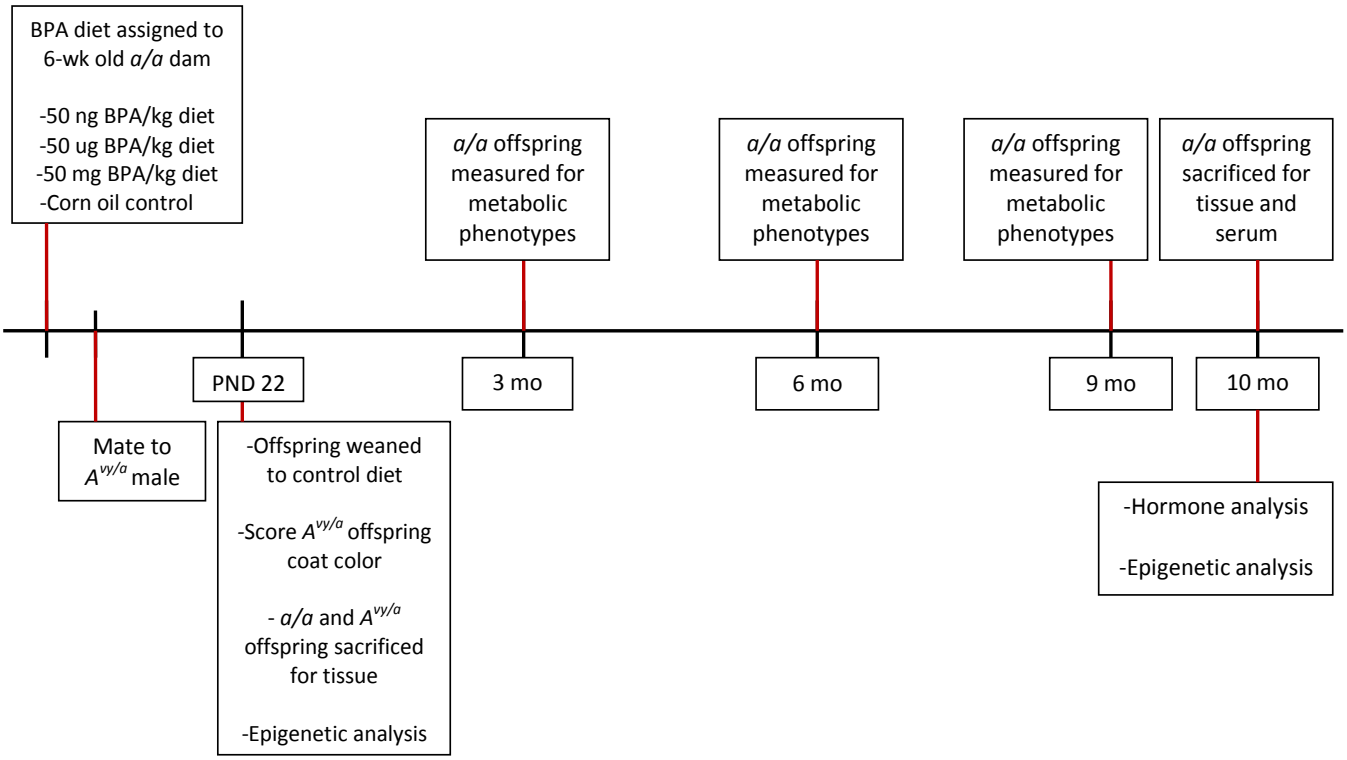
et al., 2003], but, it is important to note, all three BPA dosages capture human environmentally relevant exposure [Anderson et al., 2012; Sieli et al., 2011].

Wild-type *a/a* dams were provided with their respective diet two weeks prior to mating with 8 week old A^{vy}/a males and housed in polycarbonate-free cages with *ad libitum* access to diet and BPA-free water. The dams remained on the assigned diets throughout mating, pregnancy and lactation, after which offspring were weaned and fed the standard phytoestrogen-free control diet. This mating scheme produces approximately 50% *a/a* genotype offspring and 50% A^{vy}/a offspring. At PND 22, offspring were weighed and changed to the corn oil control diet. The coat color of all the A^{vy}/a offspring was recorded by a single observer at this time. A subset of A^{vy}/a and *a/a* wild-type mice was sacrificed at PND 22 for epigenetic analysis. A second subset of *a/a* wild-type animals was followed until 10 months of age. At 10 months of age the second subset was sacrificed for hormone and epigenetic analyses (**Figure 1.1**).

1.3.2 Life-course and Epigenetic Analysis

In the following chapters, the murine model, described in the previous section, is utilized to test the effects of developmental BPA exposure on metabolic and hormonal phenotypes throughout the life-course when the offspring are 3, 6, and 9 months of age. They undergo extensive phenotyping at the Animal Phenotyping Core at the Michigan Nutrition Obesity Research Center (MNORC) the University of Michigan. DNA methylation is explored using global methylation assay measuring CCGG sites and a candidate gene approach measuring methylation levels at seven candidate genes at PND 22, and in an epigenome-wide approach used to discovery novel regions of altered methylation at 10 months in BPA exposed offspring. Furthermore, DNA methylation is assessed as the biological target (mediator) in the development of metabolic and hormonal outcomes following perinatal BPA exposure (**Figure 1.1**).

Figure 1.1 Perinatal BPA Exposure and Life-Course Phenotyping Experimental Design



Experimental paradigm displaying bisphenol A dietary exposure, mating scheme, PND 22 epigenetic analyses, life-course metabolic phenotyping, hormone profiles, and 10 month epigenetic analyses.

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CHAPTER 2

Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life-course

2.1 Abstract

The development of adult-onset diseases is influenced by perinatal exposure to altered environmental conditions. One such exposure, bisphenol A (BPA), has been associated with obesity and diabetes, and consequently labeled an obesogen. Using an isogenic murine model, we examined the effects of perinatal exposure through maternal diet to 50 ng (n=20), 50 µg (n=21), or 50 mg (n=18) BPA/kg diet, as well as controls (n=20) on offspring energy expenditure, spontaneous activity, and body composition at 3, 6, and 9 months of age, and hormone levels at 9 and 10 months of age. Overall, exposed females and males exhibited increased energy expenditure (P<0.001 and 0.001, respectively) throughout the life-course. In females, horizontal and vertical activity increased (P=0.07 and 0.06, respectively) throughout the life-course. Generally, body composition measures were not different throughout the life-course in exposed females or males (all P>0.44), although body fat and weight decreased in exposed females at particular ages (all P<0.08). Mg exposed females had improved glucose, insulin, adiponectin, and leptin profiles (all P<0.10). Thus, life-course analysis illustrates BPA is associated with hyperactive and lean phenotypes. Variability across studies may be attributable to differential exposure duration/timing, dietary fat/phytoestrogen content, or lack of sophisticated phenotyping across the life-course.

2.2 Introduction

Human exposure to bisphenol A (BPA), the monomer comprising polycarbonate plastic and epoxy-resin, is widespread, and several studies have reported detectable levels of total urinary BPA in a majority of individuals in populations around the world, including the United States, China, and Korea [Calafat et al., 2008; Zhang et al., 2011]. BPA is produced in high volumes and found in consumer products, such as food and water containers, baby bottles, receipt paper, dental composites, and metal can linings. BPA can leach from consumer products, which presents many opportunities for human exposure, most commonly via ingestion, and to a lesser extent via inhalation and dermal routes [Vandenberg et al., 2010].

BPA can mimic or antagonize endogenous hormones, and subsequently perturb endocrine function [Gould et al., 1998; Kuiper et al., 1998]. For example, BPA has a strong binding affinity for the trans-membrane estrogen receptor (ER), G protein-coupled receptor 30 (GPR30), and estrogen related receptor gamma (ERR γ) [Takayanagi et al., 2006; Thomas et al., 2006]. BPA can activate transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and the aryl hydrocarbon receptor (AhR) [Kruger et al., 2008; Sui et al., 2012]. BPA has been labeled an environmental obesogen [Decherf et al., 2011; Heindel et al., 2009], a xenobiotic chemical contributing to obesity and related phenotypes, due to its ability to interfere with the ERs α and β in adipose and pancreatic tissues and to exert antagonistic effects on the thyroid hormone receptor [Gould et al., 1998; Kuiper et al., 1998; Moriyama et al., 2002]. As these receptor pathways play an important role in gene regulation, BPA may influence normal differentiation and maturation processes during embryonic and fetal development, predisposing individuals to chronic disease such as obesity throughout the life span [Bateson et al., 2004; Jirtle et al., 2007].

Animal models of perinatal BPA exposure provide insight into potential alterations this chemical elicits on metabolic homeostasis [Heindel et al., 2009; Rubin et al., 2009]. Male offspring developed insulin resistance and glucose intolerance as adults when exposed to 10 µg BPA/kg body weight from gestational day (GD) 9-16 [Alonso-Magdalena et al., 2010]. *In utero* BPA exposure was associated with over-expression of lipogenic genes in rats, resulting in enhanced adipogenesis of white adipose tissue measured at post-natal day (PND) 21 [Somm et al., 2009]. In human adipose tissue explants, BPA inhibited the release of adiponectin and stimulated production of cytokines interleukin-6 and tumor necrosis factor α, common pro-inflammatory markers found in overweight/obese individuals [Hugo et al., 2008]. In human epidemiological studies, Lang *et al*. found a positive association between adult BPA urinary levels and type 2 diabetes and cardiovascular disease [Lang et al., 2008], and recent data correlated BPA levels and obesity in children [Trasande et al., 2012]. Collectively, this work suggests that BPA may be a contributing factor to the obesity epidemic [Newbold, 2010].

Despite the increasing evidence describing BPA as an obesogen, another proposed target of BPA involves impairment of central nervous system (CNS) development [Masuo et al., 2011]. Animal studies revealed that perinatal BPA exposure was positively associated with spontaneous activity, aggression, and altered cognitive function [Braun et al., 2011; Kundakovic et al., 2011]. Zhou *et al.* reported that perinatal BPA exposure was associated with hyperactivity and decreased attention mediated by dopaminergic enhancement [Zhou et al., 2011]. Further animal models demonstrated that BPA targets the CNS in early development [Matsuda et al., 2010] and resulting behavioral outcomes correlated to human clinical disorders such as attention-deficit and hyperactivity disorder (ADHD) and aggression. These human clinical disorders have also been

found to be associated with maternal BPA exposure in human epidemiological studies [Braun et al., 2009; Zhou et al., 2011].

Considering the evidence from animal and human models of altered health outcomes resulting from early life BPA exposure, the aim of the present study was to assess the effects of *in utero* and early post-natal exposure to multiple physiologically relevant BPA levels on spontaneous activity, energy expenditure, body composition, and hormones/adipokines by incorporating sophisticated animal phenotyping at three time-points throughout the murine life-course. Herein we report increased energy expenditure and spontaneous activity, decreased body weight and fat, and altered hormonal parameters across BPA exposed offspring. Effects were most prevalent and observed across all doses in female offspring. Males also exhibited effects, although they were not consistently observed across all outcomes and exposure groups.

2.3 Materials and Methods

2.3.1 Animals and Diets

Mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity for the viable yellow agouti (A^{vy}) allele for over 220 generations, resulting in a genetically invariant background [Waterland et al., 2003]. Virgin wild-type *a/a* dams, 6 weeks of age, were randomly assigned to one of four phytoestrogen-free AIN-93G diets (diet 95092 with 7% corn oil substituted for 7% soybean oil; Harlan Teklad, Madison, WI): 1) standard diet (n=11 litters); 2) standard diet supplemented with 50 ng BPA/kg diet (n=14 litters); 3) standard diet supplemented with 50 µg BPA/kg diet (n=9 litters); 4) standard diet supplemented with 50 mg BPA/kg diet (n=13 litters). All diet ingredients were supplied by Harlan Teklad except BPA, which was supplied by NTP (National Toxicology Program, Durham

NC). The mg dosage is an order of magnitude lower than the dietary administered maximum non-toxic threshold in rodents (200 mg/kg body weight/day) [Takahashi et al., 2003], but, it is important to note, as previously reported all three BPA dosages capture human physiologically relevant exposure [Anderson et al., 2012].

Wild-type *a/a* dams were provided with their respective diet two weeks prior to mating with 8 week old A^{vy}/a males and housed in polycarbonate-free cages with *ad libitum* access to diet and BPA-free water. The dams remained on the assigned diets throughout pregnancy and lactation, after which offspring were weaned and fed the standard phytoestrogen-free control diet. This mating scheme produces approximately 50% *a/a* genotype offspring and 50% A^{vy}/a offspring. At PND 22, offspring were weighed and changed to the corn oil control diet. For this study, a subset of *a/a* wild-type animals, representing approximately 1 male and 1 female per litter were followed until 10 months of age: 1) standard diet (n=20 offspring); 2) 50 ng BPA/kg diet (n=20 offspring); 3) 50 µg BPA/kg diet (n=21 offspring); 4) 50 mg BPA/kg diet (n=18 offspring).

Animals used in this study were maintained in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were treated humanely and with regard for alleviation of suffering. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals.

2.3.2 Life-course Evaluation

Only wild-type *a/a* offspring were followed for life-course animal phenotyping to exclude bias from metabolic disturbance induced by the epigenetically-regulated A^{vy} allele in A^{vy}/a offspring [Miltenberger et al., 1997; Morgan et al., 1999]. These *a/a* offspring were housed

with same-sex A^{vy}/a siblings to avoid physiologic disturbances induced by singly housed animals. At 3, 6, and 9 months of age, energy expenditure, spontaneous activity, food intake, and body composition measurements were completed on *a/a* offspring at the Animal Phenotyping Core at the Michigan Nutrition and Obesity Research Center (MNORC, Ann Arbor, MI). At the Animal Phenotyping Core, offspring were singly housed and acclimated for a 7-day period in a 12-hour dark/light cycle. Proceeding the 7-day acclimation period, offspring were measured for energy expenditure (oxygen consumption, oxygen consumption for lean body mass, and carbon dioxide production) sequentially every 5-seconds in 20-minute intervals throughout a 72-hour period using an open-circuit indirect calorimeter (Columbus Instruments Comprehensive Lab Animal Monitoring System , Columbus Instruments, Columbus, OH). The respiratory exchange ratio was measured by taking the ratio of carbon dioxide production and oxygen consumption. Also during the 72-hour period of testing, spontaneous activity was measured in three axes, including ambulatory (walking), total horizontal (ambulatory plus additional horizontal movement), and vertical dimensions. Motion was detected every second in 20-minute intervals using IR photobeam technology where the interruption of a single IR beam tallied as a "count." Additionally, *ad libitum* food intake was measured using powdered food and a precision scale in 20-minute increments. After completion of the 72-hour indirect calorimetry testing, body composition was evaluated on conscious mice through NMR (Minispec LF90II, Bruker Optics, Billerica, MA) imaging, distinguishing fat tissue mass, lean tissue mass, and body fluids. Body weight was measured before initiation of the experiments at each life-course time-point.

2.3.3 Hormone Panel

Following the 72-hour period of CLAMS at the 9 month visit to the MNORC, animals were fasted for a 5-hour period and subsequently subjected to an oral glucose tolerance test (OGTT). Each animal received a glucose dose of 2 g/kg body weight via oral gavage. Glucose and insulin levels were measured at baseline, 15, 30, 60, and 120 minutes via glucometer (Acucheck, Roche) and ELISA (Millipore, St. Charles, MO), respectively (MNORC, Ann Arbor, MI). At 10 months of age, the animals were sacrificed. Females were sacrificed on the first day of estrus according to vaginal cytology [Becker et al., 2005]. Serum was collected by a hepatic vein snip. Adiponectin and leptin serum levels were measured via ELISA according to the manufacturer's instructions (Millipore, St. Charles, MO).

2.3.4 Data Analysis

Life-course energy expenditure, spontaneous activity, and food intake were evaluated at 3, 6, and 9 months of age over a 72-hour (3-day) period in 20-minute intervals. Data were collapsed (averaged) to light and dark cycles, and mixed linear regression models for repeated measures, adjusted for sex and light cycles, were estimated. Body composition was evaluated at the end of the 72-hour assessment period at 3, 6, and 9 months of age; a mixed linear regression model was estimated, adjusting for sex. The mixed linear regression models incorporated the correlation within litters with a random intercept, and correlation among repeated measures within individual mice using an autoregressive correlation structure chosen using the maximum likelihood ratio test. Models were evaluated with sex-exposure, time-exposure, and sex-time-exposure interaction terms. As a sensitivity analyses, separate models were run for each day of the 3-day assessment period for energy expenditure, spontaneous activity, and food intake at all ages (3, 6, and 9 months of age) in order to determine differences in effects across day 1 through

day 3 of assessment. Hormone analyses were completed by using a repeated measures model accounting for within litter correlation. The homeostatic model assessment of insulin resistance (HOMA-IR) was performed on fasting glucose and insulin levels. Results were considered significant at $P \leq 0.05$ and marginally significant when $P > 0.05$ and ≤ 0.10. All statistical analyses were completed using SAS software version 9.2 (Cary, NC).

2.4 Results

As previously reported, dietary physiologically relevant perinatal BPA exposure at 50 ng (n=14 litters, 107 total offspring), 50 µg (n=9 litters, 67 total offspring), or 50 mg (n=13 litters, 91 total offspring) BPA/kg diet did not significantly influence litter size (P=0.44), survival (P=0.82), genotypic ratio (P=0.46), or sex ratio (P=0.16) compared to control offspring (n=11 litters, 86 total offspring) (**Table 2.1**) [Anderson et al., 2012]. BPA exposure was significantly associated with lower wean weight of ng exposed offspring (P=0.03) but not µg or mg exposed offspring (**Table 2.1**) [Anderson et al., 2012].

For the analysis of physiological effects across the life-course at 3, 6, and 9 months, we analyzed offspring exposed to 50 ng (n=20), 50 µg (n=21), or 50 mg (n=18) BPA/kg diet, as well as controls (n=20). Due to non-monotonic and/or non-linear trends across the life span within BPA dose groups, we report values for each exposure group (ng, µg, and mg) individually in comparison to the control group at three time-points across the life span. In addition, we report the general (overall) significance of each outcome across exposure groups over time.

2.4.1 Energy Expenditure

Overall, oxygen consumption differs following perinatal dietary exposure to all levels of BPA across the life-course for females and males (P<0.001 and 0.001, respectively). Female offspring exposed to 50 ng BPA/kg developed increased oxygen consumption at 6 and 9 months of age with an average of 5156 and 4526 ml/kg/hr compared to an average of 3814 and 3749 ml/kg/hr in controls, respectively (P=0.09 and p<0.001, respectively) (**Figure 2.1A**). Females exposed to 50 µg BPA/kg had an average oxygen consumption of 4556 and 4301 ml/kg/hr versus 4152 and 3814 ml/kg/hr in the controls at 3 and 6 months of age, respectively (P=0.06 and 0.02, respectively) (**Figure 2.1A**). Females exposed to 50 mg BPA/kg had an average oxygen consumption of 4783 and 4584 ml/kg/hr versus 4152 and 3814 ml/kg/hr in the controls at 3 and 6 months of age, respectively (P=0.004 and P<0.001, respectively) (**Figure 2.1A**). At 9 months of age, males had oxygen consumption levels of 3713 and 3574 ml/kg/hr when exposed to 50 µg or mg BPA/kg, respectively compared to 3052 ml/kg/hr in controls (P=0.002 and 0.02, respectively) (**Figure 2.1A**). Additionally, oxygen consumption corrected for lean body mass was measured to avoid bias from inactive fat mass [Osman et al., 2000] (**Figure 2.1B**). Overall, oxygen consumption corrected for lean body mass was significantly different across exposure groups over the life span in females and males (P<0.001 and 0.001, respectively) (**Figure 2.1B**).

Carbon dioxide production was different across exposure groups over the life-course in females and males (P<0.001 and 0.001, respectively). Females exposed to 50 ng BPA/kg had average carbon dioxide production levels of 3897 and 3736 ml/kg/hr at 6 and 9 months of age versus 3487 and 3264 ml/kg/hr in the controls, respectively (P=0.07 and 0.05, respectively) (**Figure 2.1C**). Females exposed to 50 µg BPA/kg had average carbon dioxide production of 3692 ml/kg/hr versus 3264 ml/kg/hr in controls at 9 months of age (P=0.07) (**Figure 2.1C**). At 3 and 6 months of age, females exposed to 50 mg BPA/kg developed average carbon dioxide

production levels of 3652 and 4113 ml/kg/hr versus 4057 and 3486 ml/kg/hr in controls, respectively (P=0.10 and 0.009, respectively) (**Figure 2.1C**). Males exposed to 50 µg and mg BPA/kg developed an average carbon dioxide production of 5858 and 4021 ml/kg/hr at 3 months of age, respectively, compared to 3391 ml/kg/hr in controls (P=0.04 and 0.008, respectively) (**Figure 2.1C**). Respiratory exchange ratio was calculated to determine the source of energy metabolism used by the exposed offspring in comparison to the controls across different time-points over the life-course. No significant differences were observed. Additionally, the control group displays a natural decline in energy expenditure throughout the life-course (**Figure 2.1A, B, and C**), as noted in previous work [Goodell et al., 1998].

2.4.2 Spontaneous Activity

Horizontal activity was different across the BPA dosages over the life-course in females, but not in males (P=0.07 and 0.58, respectively). Female offspring exposed to 50 ng BPA/kg had an average horizontal activity of 2976 and 3067 counts/hr at 3 and 9 months of age compared to 2483 and 2591counts/hr in controls, respectively (P=0.04 and 0.05, respectively) (**Figure 2.2A**). At 9 months of age females exposed to 50 µg or mg BPA/kg displayed an average horizontal activity of 3241 and 3447 counts/hr, respectively, compared to 2591 counts/hr in control offspring (P=0.007 and P<0.001, respectively) (**Figure 2.2A**). Males exposed to 50 µg or mg BPA/kg had an average of 2740 and 2906 counts/hr in horizontal activity, respectively, compared to 2196 counts/hr in controls at 3 months of age (P=0.02 and 0.003) (**Figure 2.2A**).

The trend in ambulatory activity was not different over the life span across the exposure groups in females, but the trend was different males (p = 0.36 and 0.001, respectively). However, at 3 and 9 months of age, female offspring exposed to 50 ng BPA/kg exhibited average

ambulatory movement of 1740 and 1739 counts/hr compared to 1281 and 1372 counts/hr in controls, respectively, ($p = 0.01$ and 0.05, respectively) (**Figure 2.2B**). Also at 3 and 9 months of age, 50 µg BPA/kg exposed female offspring showed an average of 2180 and 1869 counts/hr in ambulatory activity compared to 1281 and 1372 counts/hr in controls ($p < 0.001$ and $p = 0.009$, respectively) (**Figure 2.2B**). Finally, at 9 months of age 50 mg exposed female offspring displayed average ambulatory activity of 1981 counts/hr versus 1372 counts/hr in controls ($p = 0.002$) (**Figure 2.2B**).

Vertical activity was different across the exposure groups throughout the life-course in females, but the trend was not different in males over time (P=0.06 and 0.87, respectively). Females exposed to 50 ng BPA/kg dietary exposure had average vertical activity of 1202 counts/hr at 9 months versus 874 counts/hr in controls (P=0.07) (**Figure 2.2C**). At 3, 6, and 9 months of age females in the µg exposed group displayed average increase vertical activity of 1086, 1312, and 1501 counts/hr versus 749, 987, and 874 counts/hr displayed by controls, respectively (P=0.06, 0.06, and P<0.001, respectively) (**Figure 2.2C**). At 3, 6, and 9 months of age, females exposed to 50 mg BPA/kg displayed average vertical activity of 1210, 1453, and 1379 counts/hr versus 749, 987, and 874 counts/hr displayed by controls, respectively (P=0.01, 0.01, and 0.007, respectively) (**Figure 2.2C**). Vertical activity in mg exposed males at 3 months of age was 1267 counts/hr compared to 750 counts/hr in controls (P=0.004) (**Figure 2.2C**). Additionally, the control group displays a natural decline in spontaneous activity levels throughout the life-course (**Figure 2.2A, B, and C**), as previously observed [Stern et al., 2012].

2.4.3 Food Intake

Food intake was different over time across the exposure groups in females and males (P=0.04 and 0.10, respectively). Females exposed to 50 ng BPA/kg had an average food intake

of 2.12 g versus 2.40 g in controls at 6 months of age (P=0.05) (**Figure 2.3**). Females exposed to 50 µg BPA/kg had average intake of 2.04 and 2.08 g at 6 and 9 months of age versus 2.44 and 2.36 g in controls, respectively (P=0.02 and 0.10, respectively) (**Figure 2.3**). At 3 months of age the mg exposed females had average intake of 2.15 g versus 2.56 g in the controls (P=0.06) (**Figure 2.3**). At 9 months of age, ng and µg exposed males exhibited average food intake of 2.90 and 2.34 g, respectively, versus 2.63 g in controls (P=0.10 and 0.08, respectively) (**Figure 2.3**).

2.4.4 Day to Day Variability Assessment

Energy expenditure, spontaneous activity, and food intake were measured over a 72-hour period (3-days) at each age of assessment (3, 6, and 9 months). The mice underwent a 7-day acclimation period prior to the onset of the 3-day phenotypic assessment, but there remained a possibility of variability among results from day 1 through 3 of testing. Thus, each day was measured separately to determine whether a particular day primarily contributed to differences between exposure and control groups. We found that differences among exposure groups versus the control group remain statistically significant from day 1 through 3 of testing. For example, mg exposed females had significantly higher oxygen consumption at 6 months of age on days 1, 2, and 3 of phenotypic assessment (P=0.02, 0.03, and 0.005, respectively) (**Figure 2.4**), and µg exposed females had significantly higher ambulatory activity at 3 months of age on days 1, 2, and 3 of phenotypic assessment (P=0.02, P<0.001, and 0.001, respectively) compared to controls. Due to homogeneous differences across the 3 days of assessment, we choose to focus on the average measurements across the total 3-day (72-hour) period of testing at each age of assessment, as reported above.

2.4.5 Body Composition

Overall, body composition measures were not significantly different over the life-course across multiple BPA doses in females and males (all $P>0.10$), but significant differences occurred in particular exposure groups and time-points when compared to controls, especially in females. At 6 and 9 months of age, ng exposed females displayed an average body weight of 27.2 and 28.4 g versus 30.9 and 32.1 g in controls, respectively ($P=0.05$ and 0.06, respectively) (**Figure 2.5A**). At 6 and 9 months of age, ng exposed females had an average fat mass of 4.00 and 4.45 g versus 6.05 and 6.15 g in controls, respectively ($P=0.03$ and 0.08, respectively) (**Figure 2.5B**). At 3 months, of age µg exposed females had average body fat of 2.33 g versus 4.07 g in control offspring ($P=0.06$) (**Figure 2.5B**). At 6 months of age, mg exposed females had average body fat of 4.50 g versus 6.05 g in controls ($P=0.08$) (**Figure 2.5B**). There were no significant changes in fat mass in males (**Figure 2.5B**) or in lean body mass in all offspring across the life span (**Figure 2.5C**).

2.4.6 Hormone Panel

The mean baseline glucose and insulin levels in mg exposed females were lower compared to controls ($P=0.05$ and 0.10, respectively) (**Figure 2.6A**). The homeostatic model assessment of insulin resistance (HOMA-IR) was conducted to capture insulin sensitivity among the exposure groups. The mg exposed females displayed a HOMA index of 3.02 versus the control females of 8.12 ($P=0.10$) (**Fig. 2.6C**). The mean adiponectin levels in ng and mg exposed females were 57.3 and 47 µg/ml, respectively, compared to 31.1 µg/ml in controls ($P=0.02$ and 0.10, respectively) (**Fig. 2.6D**). Alternatively, males exposed to 50 µg BPA/kg had marginally significant lower levels of adiponectin ($P=0.06$) (**Figure 2.6D**). Serum leptin levels in

mg exposed females were 11.1 ng/ml compared to female control serum levels of 18 ng/ml (P=0.10) (**Figure 2.6E**).

2.5 Discussion

Offspring exposed to 50 ng, µg, and mg of BPA/kg of diet during gestation and lactation developed an overall increase in energy expenditure throughout their life-course, measured via indirect calorimetry in comparison to unexposed control offspring across a 72-hour period. There are limited studies exploring *in utero* environmental exposures, especially BPA, and their impact on oxygen consumption and carbon dioxide production. McKenzie *et al.* [McKenzie et al., 2007] explored heavy metal and organic compound (PAHs, PCBs, and OCPs) exposure on the metabolic rate of adult fish in different geographical species and reported that exposed cyprinid fish in the Netherlands developed an increased resting metabolic rate. In contrast to the increased energy expenditure observed in our perinatal BPA exposure offspring cohort, adult short-term BPA exposure to 100 µg/kg body weight via injection in mice resulted in decreased energy expenditure indicating that timing, duration, and route of BPA exposure may have differing effects on metabolic endpoints [Batista et al., 2012].

Corresponding to the observed increase in energy expenditure in the current study, spontaneous activity levels were increased in offspring following perinatal BPA exposure with outcomes more prominent among females. The hyperactive phenotype found in animal models is analogous to human clinical disorders like ADHD and aggression [Wolstenholme et al., 2011]. Multiple animal models have been used to assess early life exposure to BPA and its effect on activity and behavioral-related outcomes. In mice, a single oral dose of 0.32, 3.2, or 4.8 mg BPA/kg body weight at PND 10 initiated hyperactivity in males as adults [Viberg et al., 2011]. In 3 week old male rats exposed to a maternal diet of 2 µg BPA/kg body weight from GD 7 to PND

10, Zhou *et al.* [Zhou et al., 2011] reported increased activity and discovered corresponding alterations to the dopaminergic system, which is a known target in disorders like ADHD [Ishido et al., 2005]. Zebrafish larvae exposed to 0.01 and 0.1, µM of BPA for 48 hours during post-fertilization revealed hyperactivity as adults compared to unexposed controls [Saili et al.]. Furthermore, human epidemiological evidence noted positive relationships among maternal gestational urinary BPA levels and hyperactivity and aggression in children, especially in females [Braun et al., 2011; Braun et al., 2009].

In this present study, sophisticated NMR body composition analysis revealed marginally significant changes in body weight and fat mass. In a previous report, female and male rats exposed to 2.5 or 5 µg BPA/kg body weight per day from GD 6 through PND 21 had significantly lower body weights compared to controls from PNDs 0 to 21 [Ferguson et al., 2011]. Using repeated body weight measurements, CD-1 mice exposed to dietary BPA during gestation and lactation were heavier than controls at 4 weeks of age, but this association was diminished in adulthood, and the effect did not persist in offspring fed a high fat diet [Ryan et al., 2010]. Alternatively, Wei *et al.* [Wei et al., 2011] looked at perinatal exposure to several doses of BPA by oral gavage in rats. As adults, offspring developed increased body weight when exposed to 50 µg/kg perinatally, which was exacerbated when offspring were fed a high fat diet. Female offspring exposed to 70 µg BPA/kg body weight per day via maternal drinking water from GD 6 through lactation displayed increased body weight upon weaning, with a corresponding increase in white adipose tissue and expression of lipogenic genes [Somm et al., 2009].

With much attention focused on body weight and composition after early life BPA exposure, researchers have developed a natural concern of BPA's influence on insulin sensitivity

and glucose tolerance as metabolic endpoints. Within this study, our fasting glucose and insulin tolerance test revealed an increase in insulin sensitivity with further validation using the HOMA-IR index in females treated with 50 mg BPA/kg diet *in utero* and through lactation. Conflicting evidence has emerged in the literature indicating BPA exposure in early development induces early signs of type 2 diabetes and metabolic syndrome such as glucose intolerance and insulin resistance [vom Saal et al., 2012]. For instance, male rat offspring from dams fed a BPA supplemented diet during gestation exhibited glucose intolerance and insulin insensitivity at 6 months of age [Alonso-Magdalena et al., 2010]. Adult female and male rats exposed to BPA *in utero*, displayed altered glucose and insulin homeostasis which exacerbated in males on a high fat diet [Wei et al., 2011].

We found that exposure to 50 ng and mg of BPA/kg diet increased serum adiponectin levels in female adult offspring. Adiponectin enhances insulin responsiveness and glucose uptake, is mainly produced by adipose tissue, and is negatively correlated with fat mass in humans [Ahima, 2006]. Previous reports have shown that BPA inhibits the release of adiponectin from human adipose tissue suggesting that BPA may promote the development of insulin resistance [Ben-Jonathan et al., 2009]. Interestingly, the elevated levels of adiponectin in our study were seen concurrently with increased spontaneous activity and reduced fat mass in ng and mg exposed female offspring, and reduced insulin and HOMA-IR in mg exposed female offspring with modestly lower levels of glucose during an OGTT. Thus, our data suggest that perinatal exposure to BPA elevates adiponectin and this may have occurred as a consequence to hyperactivity and reduced body fat. Of note, significant effects on these endpoints were not observed in the µg dose group, indicating possible non-monotonic effects of perinatal BPA

exposure, an area of active interest and investigation [Anderson et al., 2012; Jones et al., 2012; Vandenberg et al., 2012].

We report significantly decreased serum leptin following exposure to 50 mg BPA among females and modestly lower (non-significant) leptin levels among µg and ng, and µg and mg exposed females and males, respectively. Leptin is a hormone produced by adipose tissue that is positively correlated with body fat mass and plays a key role in energy homeostasis [Ahima, 2006]. There are limited *in vivo* studies on leptin following BPA exposure. Briefly, one study has shown that perinatal BPA exposure resulted in increased serum leptin levels in both females and males on both a high fat and normal fat diet which was positively associated with body fat [Wei et al., 2011]. In another report, serum leptin levels at PND 21 were unchanged versus controls in rats exposed to 2.5 or 5 µg BPA/kg body weight per day perinatally [Ferguson et al., 2011]. In contrast to previous reports, our data proposes that decreased leptin levels are correlated with reduced body fat following perinatal BPA exposure.

Alterations in metabolic phenotypes in BPA exposed offspring were more prominent in females than males, within the current study. Other literature describes early life BPA exposure resulting in gender specific outcomes as well [Braun et al., 2011]. Early life BPA exposure can affect sexually dimorphic traits *in vivo* when normal circulating levels of androgens are needed during brain development to shape sexually distinct behaviors [Jašarević et al., 2011; Patisaul et al., 2008; Wolstenholme et al., 2011].

2.6 Conclusion

Variable outcomes across studies highlighted throughout this discussion may be attributable to non-standardized dietary fat and/or phytoestrogen content and/or inconsistent use

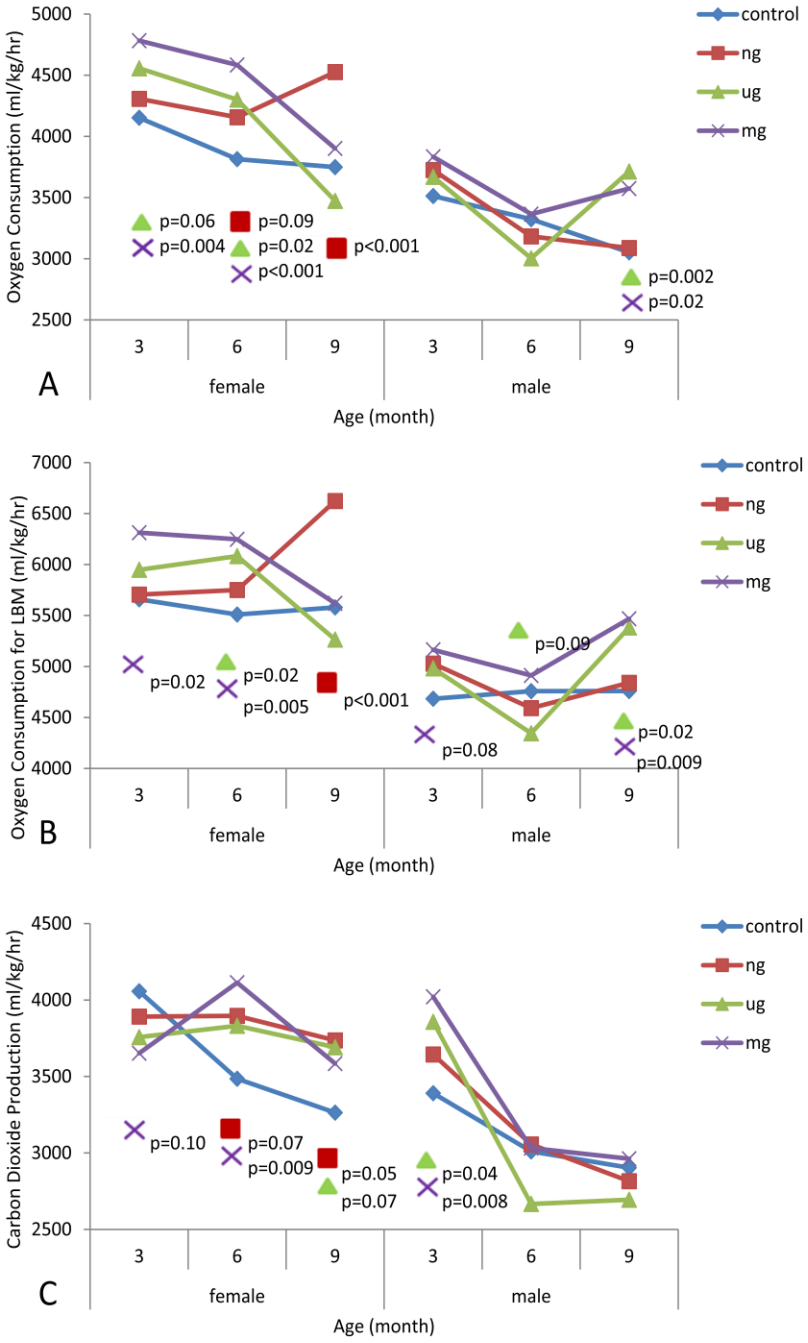
of sophisticated measurements of body composition and energy expenditure conducted at multiple time-points across the life-course. Of note, our exposure paradigm included a phytoestrogen-free and non-high fat background diet. Future studies are needed to evaluate whether diet modifies the effects of perinatal BPA exposure on metabolic and hormonal parameters throughout the life-course. Studies will need to continue to focus on timing of exposure, dose levels (particularly doses relevant to human exposure), and differences among strains and species in order to elucidate the complete phenotypic depiction of early life BPA exposure on the development of adult disease. Additionally, the effected biological pathways will need to be determined, for example, by evaluating epigenome-wide methylation and chromatin profiles, in order to understand the mechanism linking early BPA exposure to later-in-life disease risk.

Table 2.1 Litter parameters. Offspring litter size, survival rate, wean weight, genotypic ratio, and sex ratio across exposure groups

Exposure	N (litter)	Mean no. pups	Pup survival rate	Mean wean weight (g)	Mean percent *a/a* offspring	Mean percent male offspring
control	11	7.82	0.95	9.28	55	45
mg	13	7.00	0.95	9.27	51	51
µg	9	7.44	0.91	9.33	52	54
ng	14	7.64	0.93	8.65**	55	57

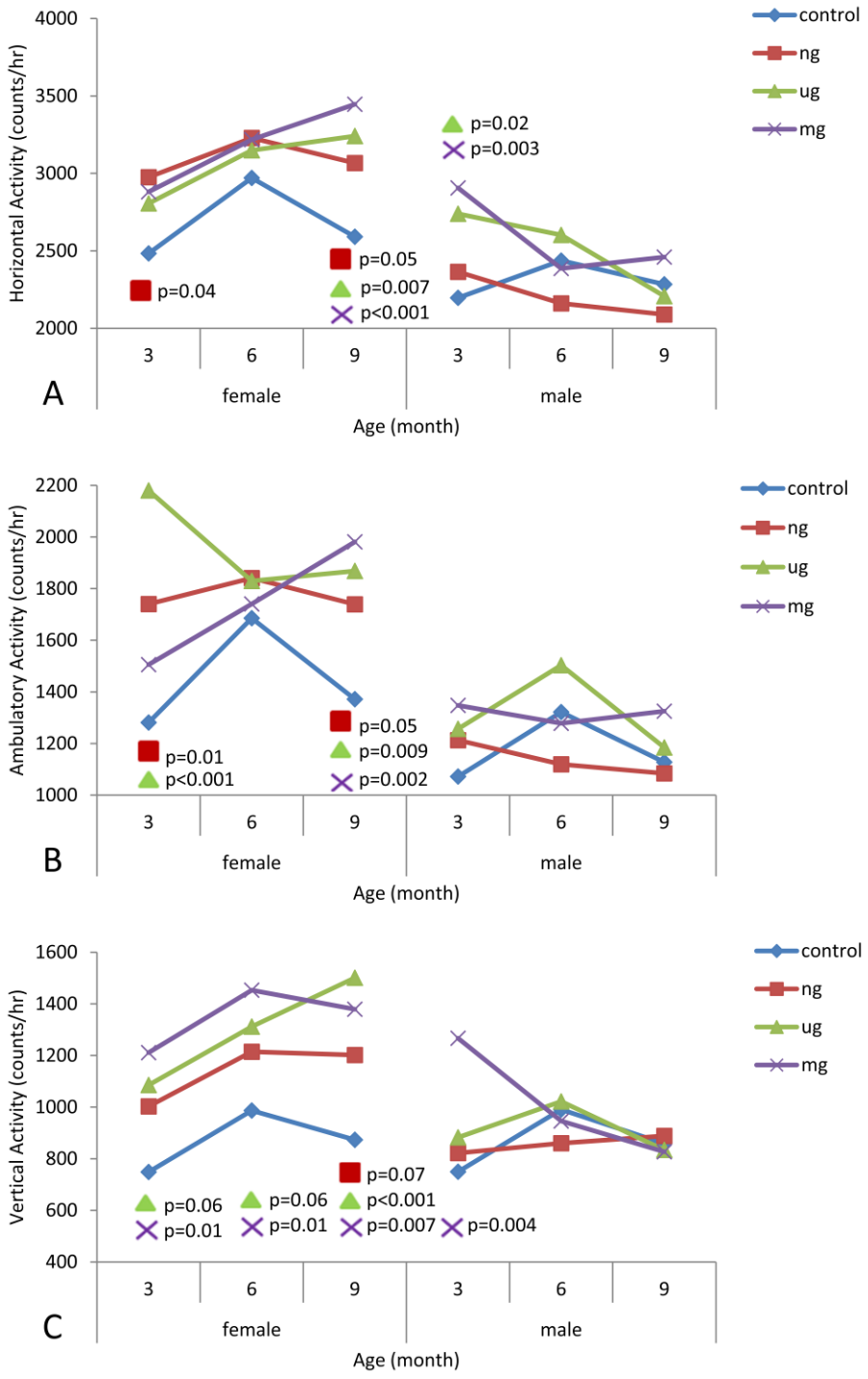
****indicates a $P < 0.05$ compared to control exposure group**

Figure 2.1 Energy expenditure (ml/kg/hr) measured by indirect calorimetry over a 72-hour period



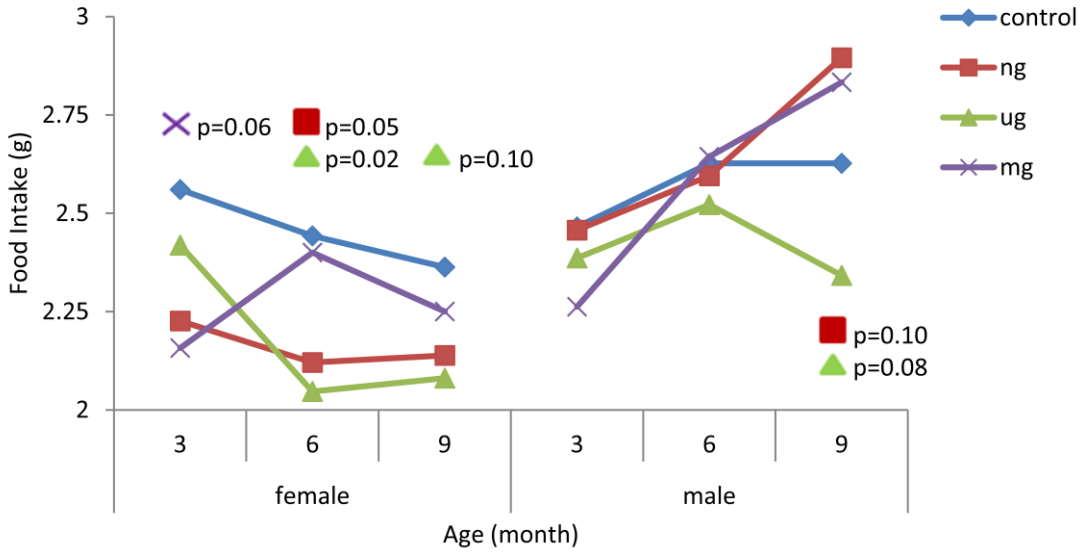
A) Oxygen consumption, **B)** carbon dioxide consumption, and **C)** carbon dioxide production across the life-course stratified by offspring sex. *P*-values represent ng (n=10 female and 10 male), µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=10 female and 10 male).

Figure 2.2 Spontaneous activity (counts/hr) measured via IR photo-beam detection average over a 72-hour period



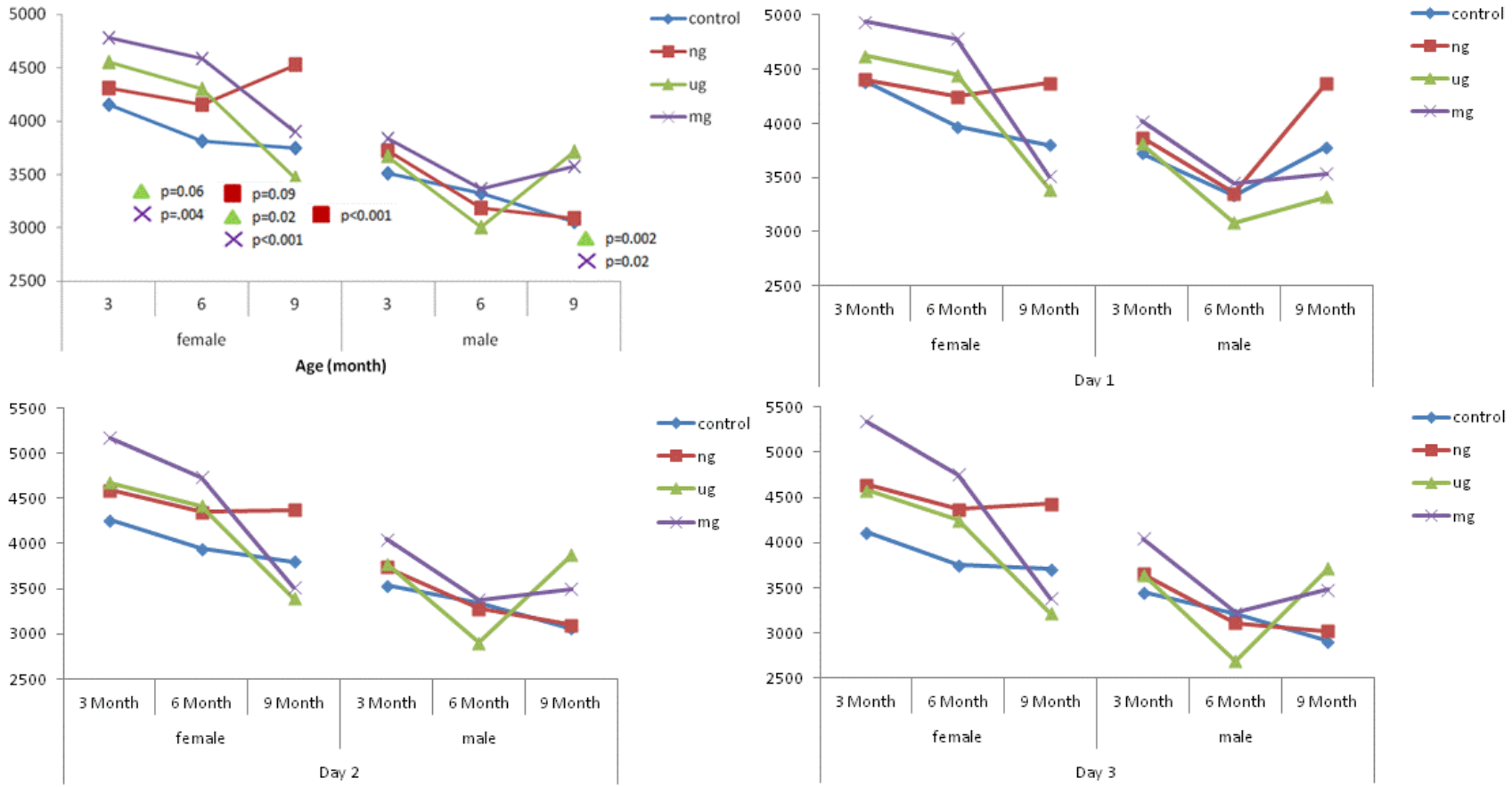
A) Horizontal, **B)** ambulatory, and **C)** vertical activity across the life-course stratified by offspring sex. *P*-values represent ng (n=10 female and 10 male), µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=10 female and 10 male).

Figure 2.3 Food intake



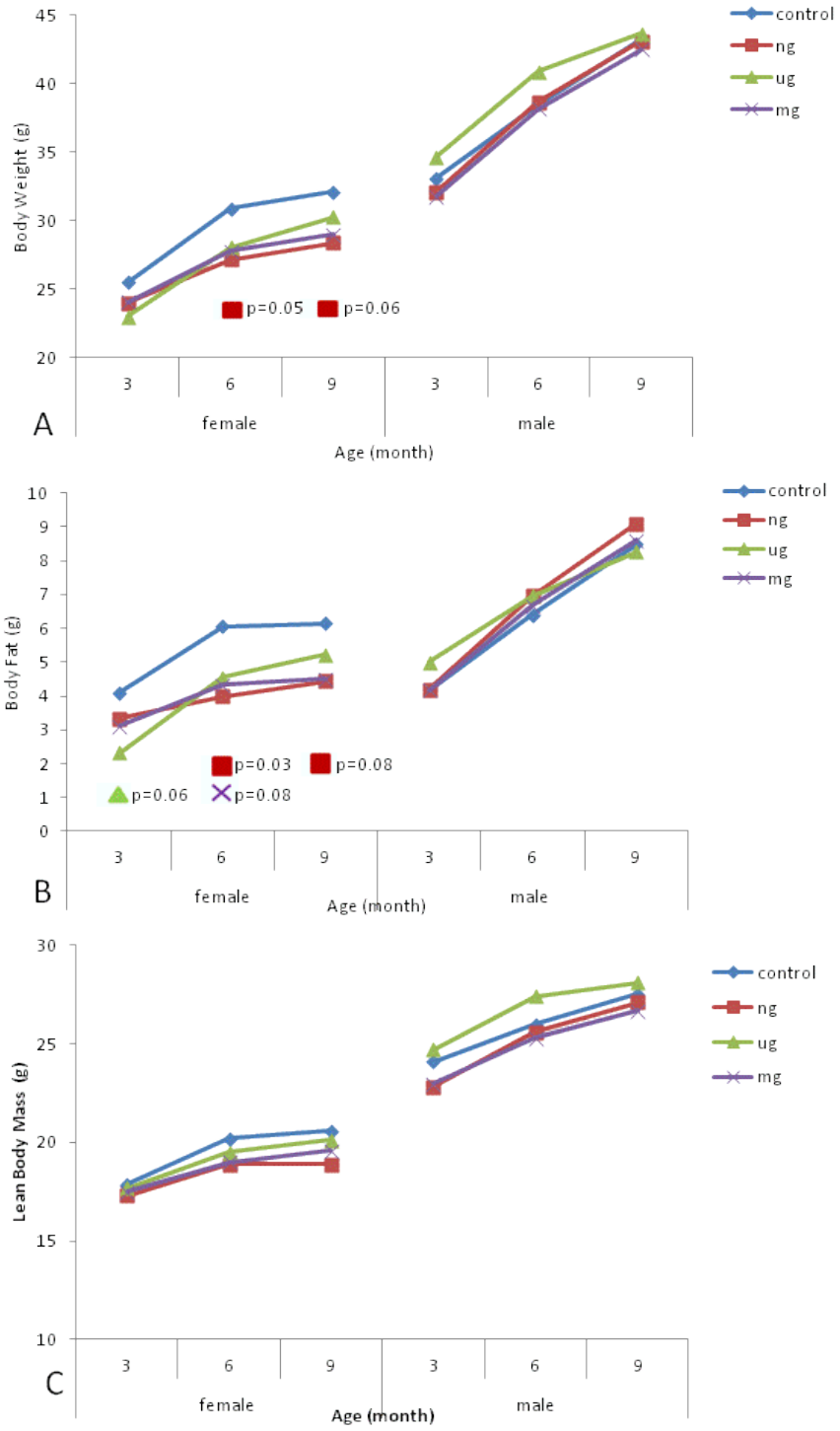
Food intake (g) across the life-course measured by powdered food on a precision scale over a 72-hour period stratified by offspring sex. *P*-values represent ng (n=10 female and 10 male), µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=10 female and 10 male).

Figure 2.4 Oxygen consumption (ml/kg/hr) as a representative example of day by day variability analysis



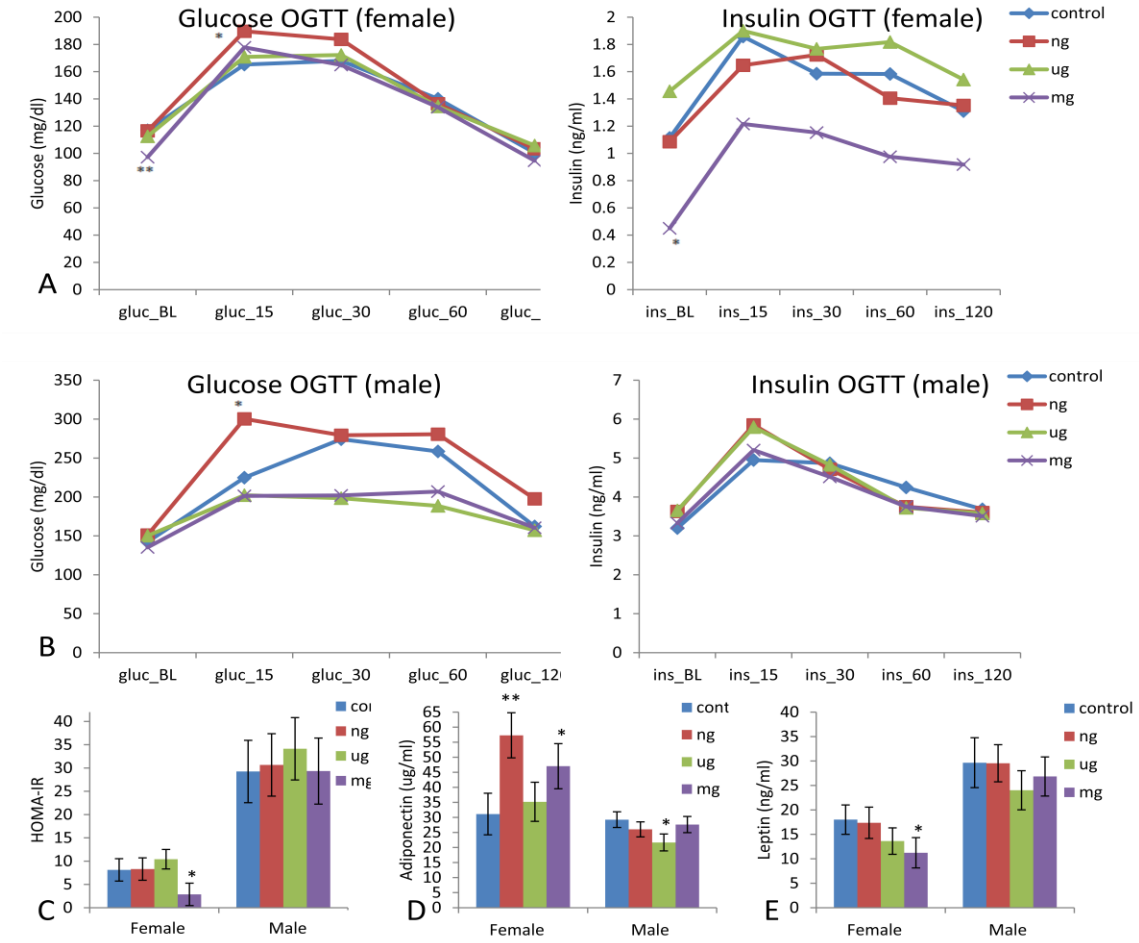
Oxygen consumption **A)** collapsed over the 72-hr period of testing across the life-course, **B)** day 1 across the life-course **C)** day 2 across the life-course, and **D)** day 3 across the life-course stratified by offspring sex. *P*-values represent ng (n=10 female and 10 male), µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=10 female and 10 male).

Figure 2.5 Body composition



Body composition (g) measured via nuclear magnetic resonance. **A)** Body weight, **B)** body fat, and **C)** lean body mass across the life-course stratified by offspring sex. *P*-values represent ng (n=10 female and 10 male), µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=10 female and 10 male).

Figure 2.6 Hormone Panel



Hormones measured at 9 or 10 months of age. **A)** Glucose (mg/dl) and insulin (ng/ml), measured via a fasting oral glucose tolerance test (OGTT) at baseline, 15, 30, 60, and 120 minutes at 9 months of age. *P*-values represent ng (n=9 female)$^{###}$, µg (n=10 female) or mg (n=9 female) BPA exposed offspring compared to control offspring (n=9 female)$^{###}$. **B)** Glucose (mg/dl) and insulin (ng/ml), measured via a fasting oral glucose tolerance test (OGTT) at baseline, 15, 30, 60, and 120 minutes at 9 months of age. *P*-values represent ng (n=10 male), µg (n=11 male), or mg (n=9 male) BPA exposed offspring compared to control offspring (n=10 male). **C)** Homeostatic model assessment of insulin resistance calculated from fasting OGTT scores. **D)** Adiponectin (µg /ml) and **E)** leptin (ng/ml) measured via ELISA at 10 months of age. *P*-values represent ng (n=9 female and 10 male)$^{###}$, µg (n=10 female and 11 male), or mg (n=9 female and 9 male) BPA exposed offspring compared to control offspring (n=9 female and 10 male)$^{###}$. **P*<0.10. ***P*<0.05. $^{###}$ng and control offspring sample size altered due to early decease.

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CHAPTER 3

Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A

3.1 Abstract

Animal studies have linked perinatal bisphenol A (BPA) exposure to altered DNA methylation, but little attention has been given to analyzing multiple human physiologically relevant doses. Utilizing the viable yellow agouti (A^{vy}) murine mouse model, we examine the effects of developmental exposure through maternal diet to 50 ng BPA/kg (n=14 litters), 50 µg BPA/kg (n=9 litters), or 50 mg BPA/kg (n=13 litters) on global and candidate gene methylation in tail and liver tissue at post-natal day 22. Global methylation analysis reveals hypermethylation in tail tissue of *a/a* and A^{vy}/a offspring across all dose groups compared to controls (n=11 litters; $P<0.02$). Analysis of coat color phenotype replicates previous work showing that the distribution of 50 mg BPA/kg A^{vy}/a offspring shifts toward yellow ($P=0.006$) by decreasing DNA methylation in tail tissue of the retrotransposon upstream of the *Agouti* gene ($P=0.03$). Maternal exposure to 50 µg or 50 ng BPA/kg, results in altered coat color distributions in comparison to control ($P=0.04$ and 0.02), but no DNA methylation effects at the *Agouti* gene are noted. DNA methylation at the CDK5 activator binding protein ($Cabp^{IAP}$) metastable epiallele in tail tissue shows hypermethylation in the 50 µg BPA/kg offspring, compared to controls ($P=0.02$). In liver tissue,

DNA methylation at the imprinted locus *H19* shows hypomethylation (*P*=0.06) in the 50 µg BPA/kg offspring, and hypermethylation at the imprinted locus *Igf2* in the 50 mg and 50 ng BPA/kg offspring (*P*=0.008 and 0.03, respectively). Comparison of exposed mouse liver BPA levels to human fetal liver BPA levels indicates that the three experimental exposures are physiologically relevant. Thus, perinatal BPA exposure affects offspring phenotype and epigenetic regulation across multiple doses, indicating the need to evaluate dose effects in human clinical and population studies.

3.2 Introduction

A growing body of work supports the developmental origins of health and disease (DOHaD) hypothesis, in which chemical and nutritional exposures early in development influence chronic disease outcomes in adulthood [Barker, 2004; Bateson et al., 2004]. Epigenetic modifications, such as DNA methylation and histone modifications, established early in development can shape susceptibility to disease, resulting in diverse phenotypes among genetically identical individuals [Rakyan et al., 2002]. For example, metastable epialleles are genes that are variably expressed due to epigenetic modifications established early in development, and thus increasingly susceptible to environmental disruption during gestation [Rakyan et al., 2002; Waterland et al., 2004]. A handful of murine metastable epialleles have been identified (A^{vy}, $Axin^{Fu}$, $Cabp^{IAP}$) in which the activity of a contraoriented intracisternal A particle (IAP) retrotransposon controls expression of an adjacent gene **(Figure 3.1)** [Druker et al., 2004; Duhl et al., 1994; Rakyan et al., 2002; Ruvinsky et al., 2001; Vasicek et al., 1997]. Importantly, the distribution of DNA methylation patterns at these metastable epialleles have been shifted following maternal exposure to nutritional and environmental factors, including

bisphenol A (BPA) [Cooney et al., 2002; Dolinoy et al., 2007; Dolinoy et al., 2006; Kaminen-Ahola et al., 2010; Waterland et al., 2003].

Accumulating work suggests that early bisphenol A (BPA) exposure increases susceptibility for adverse phenotypic outcomes via epigenetic mechanisms. BPA is a chemical used for the industrial manufacturing of polycarbonate plastics and epoxy resins. There are multiple routes of BPA exposure including ingestion, dermal absorption, and inhalation due to its widespread use in commercial products such as food and beverage containers, baby bottles, dental sealants, and receipt paper [Vandenberg et al., 2007]. Recurrent exposure to BPA is evident from detectable levels present in greater than 92% of the United States population [Calafat et al., 2008]. As an endocrine active compound, BPA can exert estrogenic activity by interfering with estrogen receptors alpha and beta, and estrogen related receptor gamma even at low exposure levels [vom Saal et al., 2006].

Early exposure to BPA may promote chronic disease development such as prostate and breast cancer, type 2 diabetes, and obesity as well as impaired brain development and behavior by altering the developing epigenome [Jirtle et al., 2007; Kundakovic et al., 2011], thus genomic loci other than metastable epialleles have examined for epigenetic disruption. For example, early developmental exposure to 10 µg BPA/kg BW/day decreased methylation of the *phosphodiesterase type 4 variant 4* gene in prostate cancer cells in adult male rats [Ho et al., 2006; Prins et al., 2008]. Additionally, *in utero* exposure to 5 mg BPA/kg BW in CD-1 mice on days 9-16 of pregnancy decreased methylation in the promoter region of *Hoxa10*, a gene involved in uterine organogenesis [Bromer et al., 2010]. Previously, our group reported a shift towards DNA hypomethylation at the viable yellow agouti (A^{vy}) and CDK5 activator binding protein ($Cabp^{IAP}$) metastable epialleles **(Figure 3.1A and B)** in offspring exposed to a relatively

high dosage of BPA (50 mg BPA/kg diet) perinatally [Dolinoy et al., 2007]. Moreover, restoration of normal methylation patterns occurred with maternal supplementation of genistein or methyl donors such as folate, choline, betaine, and vitamin B_{12} [Dolinoy et al., 2007; Dolinoy et al., 2006].

Previous epigenetics studies involving BPA have not captured the full range of human physiologically relevant exposure levels, and most attempts to elucidate the effects on the epigenome following environmental manipulations, including perinatal exposure to BPA, have been restricted in dose-response assessment. Thus, utilizing the viable yellow agouti (A^{vy}) mouse model, we examined global and candidate gene methylation patterns following perinatal exposure at three dosages (50 ng, µg, and mg of BPA/kg diet). The murine A^{vy} allele resulted from the random insertion of a murine IAP retrotransposon into the 5' end of the *Agouti* gene **(Figure 3.1A)** [Duhl et al., 1994]. Methylation of CpG sites in and near the A^{vy} IAP correlates inversely with ectopic *Agouti* expression and varies dramatically among isogenic A^{vy}/a mice, resulting in a wide array of coat colors, ranging from yellow (unmethylated) to pseudoagouti (methylated), and additionally results in adult-onset obesity (~10 weeks of age) among low methylated mice [Miltenberger et al., 1997; Morgan et al., 1999]. To our knowledge, this is the first study to utilize the A^{vy} mouse model as an epigenetic biosensor to evaluate maternal exposure to multiple, rather than single dose levels.

3.3 Materials and Methods

3.3.1 Animals and Diets

A^{vy} mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity for the A^{vy} allele for over 220 generations, resulting in a genetically

invariant background [Waterland et al., 2003]. Virgin *a/a* dams, 6 weeks of age, were randomly assigned to one of four phytoestrogen-free AIN-93G diets (diet 95092 with 7% corn oil substituted for 7% soybean oil; Harlan Teklad, Madison, WI): 1) standard diet (n=11 litters, 86 total offspring, 39 A^{vy}/a offspring); 2) standard diet supplemented with 50 ng BPA/kg diet (n=14 litters, 107 total offspring, 48 A^{vy}/a offspring); 3) standard diet supplemented with 50 µg BPA/kg diet (n=9 litters, 67 total offspring, 32 A^{vy}/a offspring); 4) standard diet supplemented with 50 mg BPA/kg diet (n=13 litters, 91 total offspring, 45 A^{vy}/a offspring). All diet ingredients were supplied by Harlan Teklad except BPA, which was supplied by NTP (National Toxicology Program, Durham NC). The mg dosage was formulated to be an order of magnitude lower than the dietary administered maximum non-toxic threshold in rodents (200 mg/kg BW/day) [Takahashi et al., 2003], while the ng and µg BPA dosages were used to potentially capture the physiologically relevant range of human exposure.

Following 2 weeks on their respective diets, at 8 weeks of age *a/a* virgin dams were mated with A^{vy}/a males, 8 weeks of age. All animals were housed in polycarbonate-free cages and provided *ad libitum* access to diet and BPA-free water. The dams remained on the assigned diets throughout pregnancy and lactation. At post-natal day 22 (PND 22), *a/a* and A^{vy}/a offspring were weighed and a tail sample was collected. Also at PND 22, a single observer visually classified A^{vy}/a offspring coat color phenotype into one of five categories based on proportion of brown fur: yellow (<5% brown), slightly mottled (between 5% and 40% brown), mottled (~50% brown), heavily mottled (between 60% and 95% brown), and pseudoagouti (>95% brown). Tail tissue was collected for analysis from all offspring.

Animals used in this study were maintained in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were

treated humanely and with regard for alleviation of suffering. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals.

3.3.2 DNA Isolation and Methylation Analysis

Total genomic DNA was isolated from PND 22 tail tissue of all *a/a* and A^{vy}/a offspring using magnetic particle-based methodology and the Maxwell 16® Instrument (Promega Corporation, Madison, WI). Total genomic DNA was isolated from PND 22 liver of all *a/a* and A^{vy}/a offspring using a standard phenol-chloroform extraction method. Briefly, about 15 mg of tissue was resuspended in 540 ul buffer ATL and is homogenized for 20 seconds at 15 Hz (TissueLyser, Qiagen). The lysate was transferred to 60 ul of Proteinase K and incubated for overnight at 50□C. After overnight incubation 12 ul of RNase A is added to lysate and incubated for 10 minutes at 37□C. Samples are extracted twice with 600 ul phenol–chloroform–isoamyl and once with 600 ul chloroform. 50μL of 3M sodium acetate is added to aqueous phase and precipitated with 1 mL ice-cold 100% ethanol. For additional precipitation, 1 mL of ice-cold75% ethanol is added to pellet twice. The pellet was air dried and resuspended with 100 ul Tris-EDTA buffer and incubated for 2 hours at 60□C with frequent mixing.

The Luminometric Methylation Assay (LUMA), a methylation-sensitive restriction enzyme digest followed by quantitative DNA methylation analysis via pyrosequencing, was utilized to analyze global DNA methylation at CCGG sites throughout the mouse genome using genomic tail DNA [Karimi et al., 2006]. Approximately 300 ng of genomic tail DNA was digested with both *Hpa*II, an endonuclease sensitive to CpG methylation, and *Msp*I, an endonuclease insensitive to CpG methylation; both endonucleases cut CCGG sites between the first and second cytosines. *Eco*RI was used in both restriction enzyme reactions as a

normalization reference [Karimi et al., 2006]. Following DNA digestion, samples were pyrosequenced in duplicate to quantify CCGG DNA methylation. The pyrosequencing output provides the incorporation of dCTP, which is directly correlated with DNA methylation. The ratio of digested sites to undigested sites was calculated and represents the percentage of genomic DNA methylation. Duplicates with measurement differences greater than 15% were omitted prior to statistical analysis.

Using the Qiagen Epitect kit automated on the Qiagen QIAcube® purification system, approximately 1 µg of genomic tail and liver DNA was treated with sodium bisulfite to allow conversion of unmethylated cytosines to uracil, read as thymine during polymerase chain reaction (PCR), whereas the methylated cytosines remain unconverted [Grunau et al., 2001]. Following bisulfite conversion, candidate gene regions of interest were amplified using HotStarTaq master mix (Qiagen Inc., Valencia, CA), forward primer (0.5 pmol) and reverse primer (0.5 pmol) in a 30 µL PCR and resolved by gel electrophoresis. PCR and sequencing primers for assays are listed in **Table 3.1**. DNA methylation of CpG sites of interest was quantified using PyroMark MD (Qiagen Inc., Valencia, CA) pyrosequencing technology. Percent methylation was computed using PyroMark software, which calculates the degree of methylation as percent 5-methylated cytosines (%5mC) over the sum of methylated and unmethylated cytosines**.** All samples were run in duplicate using the average CpG site methylation of the duplicates for statistical analysis. Sequences to analyze for pyrosequencing runs are provided in **Table 3.1**. The four CpG sites studied at the A^{vy} allele are located at nucleotide positions 306, 319, 322, and 334 of GenBank accession number AF540972.1. The four CpG sites studied at the $Cabp^{IAP}$ allele are located at nucleotide positions 44, 57, 60 and 72 of GenBank accession number BB842254. The eight CpG sites studied at the *Igf2* allele are located in the differentially

methylated region 2 (DMR2), and the four sites studied at the *H19* allele are located in the imprinted control region. The five, three, and two CpG sites studied at *HsdBeta11 Type 2, Ppar-alpha*, and *Ppar-gamma*, respectively are located 150 bp, 400 bp, 260 bp upstream of the TSS in the promoter region.

3.3.3 Statistical Analysis

The influence of gestational BPA exposure on litter size, survival, wean weight, genotypic ratio, and sex ratio was evaluated using ANOVA with Bonferroni post-hoc analysis. The distribution of the five coat color phenotypes between each exposure group was analyzed using a chi-square goodness-of-fit test, with the control coat color distribution representing the expected distribution. All comparisons resulted in cell counts with no more than 20% of cells containing fewer than 5 observations. The influence of sex, genotype and coat color on mean CpG methylation was measured using ANOVA with Bonferroni corrections. Average CpG methylation within an amplicon, site-specific CpG methylation at A^{vy} and $Cabp^{IAP}$, and global methylation among the three BPA exposed groups and the control group were evaluated by two-sample hypothesis analysis of means and ANOVA with Bonferroni correction as post-hoc analyses. Average and site-specific CpG methylation for *H19, Igf2, HsdBeta11 Type 2, Ppar-alpha, and Ppar-gamma* were evaluated using a mixed effects regression model to account for correlation among CpG sites within an amplicon using an unstructured covariance structure chosen from the maximum likelihood ratio test. Models were evaluated with a CpG site-exposure interaction term. Two additional mixed effects regression models were conducted to stratify by sex and genotype to determine their influence on methylation results. Statistical significance was defined as P-value <0.05 for all analyses. Normality of percent methylation was evaluated using

histograms and Q-Q plots. Outliers defined as having a studentized residual greater than 2.0 were excluded in the final methylation analysis. The resulting exclusion of outliers consisted of two mg exposed subjects for A^{vy} locus methylation analysis. All statistical analyses were completed using SAS software version 9.2 (Cary, NC).

3.3.4 BPA Analysis in Liver

Approximately 300-500 mg of PND 22 A^{vy}/a mouse liver tissue (n=8-11 per exposure group) was flash frozen and homogenized to fine powder over a mortar above liquid nitrogen. Homogenized tissue was transferred into a 2 mL polypropylene eppendorf tube and transported overnight on dry ice to the Wadsworth Center (New York State Department of Health, Albany, NY). Five mL of acetonitrile and 5 ng of $^{13}C_{12}$-BPA, an internal standard, were added to homogenized tissue, and the mixture was shaken for 30 minutes. Extraction with acetonitrile was repeated twice and the mixture was centrifuged at 4500 x g for 3 minutes. Combined aliquots were concentrated to near-dryness under a gentle stream of nitrogen and reconstituted with 1.5 mL of 10% dichloromethane in hexane. The sample extract was then loaded onto a Strata® NH_2 cartridge (200 mg/3 mL, Phenomenex, Torrance, CA), pre-conditioned with 5 mL of 80% methanol in acetone and 5 mL of hexane. The cartridge was washed with 5 mL of hexane and eluted with 5 mL of 80% methanol in acetone. The eluate was concentrated to 0.5 mL under a gentle stream of nitrogen resulting in the free BPA fraction.

To determine conjugated BPA, 1 mL of Milli-Q H_2O and 1 mL of 2 µL/mL β-glucuronidase (from *Helix pomatia*, 145700 units/mL, Sigma, St Louis, MO) were added to the residue from free-BPA aliquot. $^{13}C_{12}$-BPA was added and digested at 37^{o}C for 12 hours. The sample was extracted thrice with ethyl acetate (5 + 3.5 + 3.5 mL) and purified by passing

through Strata® NH_2 cartridge as described above. The final eluate was concentrated to 0.5 mL. A high-performance liquid chromatograph (HPLC) interfaced with API 2000 electrospray triple-quadruple mass spectrometry (ESI-MS/MS; Applied Biosystems, Foster City, CA) was used for the quantification of free and conjugated BPA [Padmanabhan et al., 2008]. Ten µL of the extract was injected onto an analytical column (Betasil® C18, 100 × 2.1 mm column; Thermo Electron Corporation, Waltham, MA), which was connected to a Javelin® guard column (Betasil® C18, 20 × 2.1 mm). The mobile phase flow rate was 300 µL/min. The mobile phase consisting of methanol and water started at a gradient of 25% methanol to 99% methanol in 4 minutes and was held for 10 minutes before reverting to its initial condition. The MS/MS was operated in the electrospray negative ionization mode and optimized to transmit the $[M-H]^-$ ion before fragmentation of one or more product ions. Cone voltage and collision energies were 30 V and 25 V, respectively. The capillary voltage was 4.5 KV, and desolvation temperature was 400°C. Multiple reaction monitoring transitions monitored were 227 > 212 for BPA, and 239 > 224 for $^{13}C_{12}$-BPA.

A procedural blank was analyzed with every 10 samples to check for interferences or laboratory contamination. The limit of quantitation (LOQ) of BPA was 0.1 ng/g. The LOQ was calculated as twice the concentration of the "lowest acceptable calibration standard"; the amount of sample taken for analysis and final extract volume. The mean recovery of $^{13}C_{12}$-BPA spiked into samples was 96%. Reported concentrations were corrected for the recoveries of the internal standard (isotope dilution method). BPA standards spiked into selected sample matrices and passed through the entire analytical procedure yielded a mean recovery of 101%. An external calibration curve was prepared by injecting 10 µL of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 50, and 100 ng/mL standards and the calibration coefficient was 0.99.

In order to compare mouse liver BPA concentrations to physiologically relevant levels in humans, free and conjugated BPA were measured in 51 human fetal liver samples obtained from the NIH-funded (R24 HD000836-47) Birth Defects Research Laboratory fetal tissue bank at the University of Washington, Seattle. These tissues were derived from conceptuses with gestational ages ranging from 74 to 120 days. Information provided with these tissues include gestational age and sex; occasionally race is indicated. The health status of the individuals is unknown but presumed to have been healthy and with no known chromosomal abnormalities. Prior to shipment to the University of Michigan, samples were flash frozen with liquid nitrogen and stored in polycarbonate-free tubing. Fetal liver tissues were processed for BPA levels as described above.

To ensure no contamination was introduced into the sample preparation process, a negative control of BPA-free water was processed identically as the mouse and human samples described above and resulted in free and conjugated BPA levels below the LOQ. For calculation of mean and median BPA concentrations, liver BPA levels below the LOQ were assigned a value of 0.071, which was estimated by dividing the LOQ (0.1 ng/g) by the square root of 2.

3.4 Results

3.4.1 Overview

Gestational BPA exposure at 50 ng BPA/kg (n=14 litters, 107 total offspring), 50 µg BPA/kg (n=9 litters, 67 total offspring), or 50 mg BPA/kg (n=13 litters, 91 total offspring) diet did not significantly influence litter size (P=0.84), survival (P=0.86), genotypic ratio (P=0.49), or sex ratio (P=0.16) in comparison to control offspring (n=11 litters, 86 total offspring) (**Table 3.1**). BPA exposure was, however, significantly associated with lower wean weight of ng

exposed *a/a* and A^{vy}/a offspring (mean weight 8.65 g in ng versus 9.28 g in control; *P*=0.03) but not µg or mg exposed offspring (**Table 2.1**). When PND 22 body weight analysis is restricted to *a/a* animals only, the decreased body weight in ng exposed offspring, and not in µg and mg exposed offspring, is still observed (mean weight 8.24 g in ng versus 9.15 g in control; *P*=0.01).

3.4.2 Global DNA Methylation

Global CCGG DNA methylation levels throughout the mouse genome were measured in A^{vy}/a and *a/a* offspring PND 22 tail DNA using the LUMA assay (**Table 3.2**). Mean methylation did not differ by sex (*P*=0.58), genotype (*P*=0.43), or coat color (*P*=0.93). Offspring exposed to the 50 mg BPA/kg diet demonstrated an average of 59.3% global DNA methylation in comparison to control offspring with measured percent global methylation of 51.6 (n=71 and 60, respectively; *P*=0.01). Offspring exposed to the 50 µg BPA/kg diet also exhibited significantly higher average percent global DNA methylation of 60.3 compared to controls (n=52 and 60, respectively; *P*=0.008). Lastly, the offspring exposed to a 50 ng BPA/kg diet showed significantly higher average percent global methylation of 58.8 compared to controls (n=74 and 60, respectively; *P*=0.02). There were no statistically significant differences in global methylation among the three BPA exposed groups.

3.4.3 Maternal Dietary BPA Exposure Coat Color Shift

The total number of offspring evaluated for coat color shift was among 11 litters from the corn oil control group (n=39 A^{vy}/a offspring), 13 litters from the mg BPA/kg supplemented group (n=45 A^{vy}/a offspring), 9 litters from the µg BPA/kg supplemented group (n=32 A^{vy}/a offspring), and 14 litters from the ng BPA/kg supplemented group (n=48 A^{vy}/a offspring). Perinatal BPA

exposure through maternal diet shifted the coat color distribution of A^{vy}/a offspring in a dose-dependent fashion. Maternal dietary exposure to 50 mg BPA/kg shifted the coat color distribution of genetically identical PND 22 A^{vy}/a offspring towards yellow in comparison to control offspring (P=0.006) **(Figure 3.2A).** Twenty-nine percent of the 50 mg BPA/kg exposed offspring were classified as yellow coat color compared to only 15% of control offspring. Conversely, maternal dietary exposure to 50 µg BPA/kg resulted in a statistically significant altered coat color distribution in comparison to controls (P=0.04) **(Figure 3.2B)**. Twenty-five percent of the 50 µg BPA/kg exposed offspring were classified as pseudoagouti compared to only 12% of the control offspring; however the 50 µg BPA/kg exposed offspring were also more likely to be classified as slightly mottled (31% compared to 18%). Finally, maternal dietary exposure to 50 ng BPA/kg resulted in increased incidence of slightly mottled and heavily mottled offspring compared to controls (P=0.02) but did not shift the coat color distribution towards the pseudoagouti or yellow phenotype **(Figure 3.2C).**

3.4.4 DNA Methylation at the A^{vy} and $Cabp^{IAP}$ metastable epialleles

Site-specific and average CpG DNA methylation in PND 22 tail tissue at four CpG sites (sites 6-9) in the cryptic promoter of the A^{vy} IAP was quantified using bisulfite pyrosequencing on A^{vy}/a offspring **(Figure 3.1A).** The mg exposure group (n=43 A^{vy}/a offspring) demonstrated an average methylation of 24.3% across the four CpG sites in comparison to 35.6% average methylation of the controls **(Table 3.3)** (n=38 A^{vy}/a offspring; P=0.03). Evaluation of each individual CpG site indicated a significant decrease in methylation at sites 6 (P=0.03), 7 (P=0.02) and 8 (P=0.02) in the mg exposed offspring versus control. Methylation at site 9 was marginally significantly lower (P=0.07). Average methylation across the four CpG sites of the

µg (n=32 A^{vy}/a offspring; P=0.97) and ng (n=48 A^{vy}/a offspring; P=0.79) exposed offspring did not differ in comparison to the control group **(Table 3.3)**. There were no significant differences in methylation at sites 6-9 of µg (P=0.96. 0.93, 0.99, and 0.80, respectively) and ng (P=0.79, 0.60, 0.75, and 0.99, respectively) groups in comparison to the control group.

DNA methylation in PND 22 tail tissue at four CpG sites (sites 6-9) in the $Cabp^{IAP}$ metastable epiallele [Druker et al., 2004] was measured in both A^{vy}/a and *a/a* offspring **(Figure 3.1B)**. In comparison to the control group (n=82 A^{vy}/a and *a/a* offspring), the mg exposed offspring (n=85 A^{vy}/a and *a/a* offspring) showed no difference in average methylation across the four CpG sites (83.1% versus 83.6%, respectively; P=0.64) **(Table 3.3)**. Average methylation across the four CpG sites in the µg exposed offspring (n=67 A^{vy}/a and *a/a* offspring), however, was increased compared to controls (85.8% and 83.1%, respectively; P=0.02). Site-specific methylation showed increased methylation at sites 6, 8, and 9 in the µg exposed group versus the controls (P=0.01, 0.02, and 0.04, respectively). The ng exposure group (n=107 A^{vy}/a and *a/a* offspring) displayed an average methylation across the four CpG sites of 84.3% compared to 83.1% in the control group (P=0.25). Sites 8 and 9 exhibited statistically significant increased methylation in ng exposed offspring when compared to controls (P=0.02 and 0.04, respectively).

3.4.5 Candidate Gene DNA Methylation at *Ppar-alpha, Ppar-gamma, HsdBeta11 Type 2, Igf2,* and *H19*

DNA methylation in PND 22 liver tissue at four CpG sites in the imprinted gene *H19* was measured in both A^{vy}/a and *a/a* offspring. In comparison to the control group (n=37 A^{vy}/a and *a/a* offspring), the mg and ng exposed offspring (n=40 and 46 A^{vy}/a and *a/a* offspring, respectively) showed no difference in average methylation across the CpG sites (54.4% versus 55.1% and

54.0%, respectively; *P*=0.47 and 0.72, respectively) (**Figure 3.3A**). Alternatively, the µg exposed group (n=24 A^{vy}/a and *a/a* offspring) displayed a marginally significant decrease in average methylation compared to the control group (52.2% versus 54.4%, respectively; *P*=0.06) (**Figure 3.3A**).

Also in PND 22 liver tissue, DNA methylation at eight CpG sites was measured in the imprinted gene *Igf2*. The µg exposed group (n=22 A^{vy}/a and *a/a* offspring) showed no significant changes in average methylation compared to the control group (n=36 A^{vy}/a and *a/a* offspring; 39.3% versus 40.6%, respectively; *P*=0.31) (**Figure 3.3B**). In comparison to the control group, the mg and ng exposed offspring (n=39 and 48 A^{vy}/a and *a/a* offspring, respectively) showed significant increases in average methylation across the CpG sites (40.6% versus 44.4% and 42.7%, respectively; *P*=0.008 and 0.03, respectively) (**Figure 3.3B**).

DNA methylation was significantly altered at specific CpG sites at the *HsdBeta11 Type 2* locus. In the ng exposed offspring (n=48 A^{vy}/a and *a/a* offspring) DNA methylation was significantly decreased at site 4 compared to the control group (n=36 A^{vy}/a and *a/a* offspring; 1.06% versus 2.98%, respectively; *P*=0.03) (**Figure 3.3C**). In the µg exposed offspring (n=21 A^{vy}/a and *a/a* offspring) there was a significant increase in DNA methylation at site 1) compared to the controls (5.29% versus 4.25%; *P*=0.006) (**Figure 3.3C**). There were no significant changes in average methylation in any of the exposed offspring compared to the control offspring (all *P*>0.35) (**Figure 3.3C**). Additionally, there were no significant changes in average DNA methylation or individual CpG site at the *Ppar-alpha* and *Ppar-gamma* loci (all *P*>0.10) (**Figure 3.3D and E).**

3.4.6 Liver BPA Measurements

Free and conjugated BPA concentrations were analyzed in a subset of PND 22 mouse liver samples (~1 pup per litter) from each BPA exposure and control group as well as in 51 human fetal liver samples **(Table 3.4).** Total BPA (free plus glucuronide-conjugated) concentrations measured in the 50 mg BPA/kg exposed mice ranged from 9.46 to 870 ng/g (mean=441; median=472; n=9). Total BPA in animals exposed to 50 µg BPA/kg ranged from below LOQ to 11.3 ng/g (mean=2.02; median=0.56; n=10). BPA concentrations in the liver from mice exposed to 50 ng BPA/kg ranged from below LOQ to 13.0 ng/g (mean=2.78; median=0.31; n=11). Total BPA in the control group ranged from below LOQ to 11.5 ng/g (mean=4.26; median=4.24; n=10). To compare mouse liver BPA concentrations to physiologically relevant doses in humans, fetal human liver tissues were also analyzed for free and glucuronide-conjugated BPA. Total BPA concentrations in human fetal liver ranged from below LOQ to 96.8 ng/g (mean=10.8, median=3.39; n=51). The overlap between mouse liver BPA levels and human fetal liver BPA levels indicates that the experimental approach employed for dietary animal exposure captures a relevant and full range of human BPA exposure.

3.5 Discussion

In this chapter, dose-dependent phenotypic and epigenetic responses following maternal dietary exposure to three levels of BPA were reported. First, a decrease in PND 22 wean body weight in *a/a* and A^{vy}/a offspring exposed to 50 ng/kg diet of bisphenol A (BPA) versus control offspring was observed. This association persists when analysis is restricted to *a/a* offspring alone, indicating that this effect is not associated with the epigenetically-controlled adult onset obesity associated with A^{vy}/a offspring, but rather manifests as a result of perinatal BPA exposure. Body weight differences were not detected in offspring exposed to either 50 µg or 50 mg BPA/kg diet indicating a non-monotonic dose response of wean weight and, corroborating

previous studies using multiple doses of BPA with non-linear outcomes [Honma et al., 2002; Rubin et al., 2001]. Non-monotonic effects of BPA on DNA methylation suggest that BPA, and endocrine disruptors in general, can effect different mechanisms involved in epigenetic regulation and should be further investigated.

Global methylation of the mouse genome assessed using the LUMA assay reveals a significant increase in methylation across all BPA exposure groups in comparison to controls. This assay provides a measure of methylation at CCGG sites throughout the entire genome regardless of location, representing the degree to which the genome is globally methylated. The LUMA assay has been extensively employed in analysis of human cancers [Deneberg et al., 2010; Lee et al., 2008; Poage et al., 2011]. There are limited studies, however, exploring environmental and/or nutritional exposures and their impact on global methylation [Gallou-Kabani et al., 2010]. Recently, using a mouse model, Gallou-Kabani *et al.* [2010] associated maternal high fat diet with decreased placental tissue DNA methylation in female offspring . The global decrease in CCGG methylation was not associated with decreased methylation at LINE-1 or B1 repetitive elements. Gene specific methylation at the *Igf2r* gene in female offspring exposed to high fat diet, on the other hand, was increased. Thus, it is important to note that the LUMA assay is restricted to methylation of CCGG sequences throughout the genome and is not necessarily representative of physiologically-induced local changes at candidate genes or repetitive content derived from transposable elements such as LINE-1 and B1.

We also note dose-dependent shifts in the coat color distribution of genetically identical A^{vy}/a offspring exposed to a 50 mg, µg, or ng/kg diet of BPA perinatally. The coat color distribution of offspring exposed to a 50 mg/kg diet of BPA displays a shift towards the yellow obese phenotype, reproducing our 2007 single dose study results [Dolinoy et al., 2007], while the

µg dose offspring displays a shift towards the pseudoagouti lean coat color phenotype. Average methylation at the A^{vy} locus of the mg exposed offspring is significantly decreased in comparison to the control group, providing epigenetic validation of the coat color distribution shift. In contrast, average methylation at the A^{vy} locus of the µg exposure group was not statistically significant. An excess of categorization as slightly mottled A^{vy}/a offspring may have offset a hypermethylation response among the µg exposure group when compared to the control group. Increased methylation in µg offspring compared to control offspring was, however, detected at the $Cabp^{IAP}$ metastable epiallele, signifying that perinatal exposure to BPA at this dose increases methylation at this particular epigenetically labile locus. Taken together, these results 1) indicate that methylation at more than one locus is variable after perinatal exposure to BPA, 2) strengthen the evidence for non-monotonic dose-dependent effects of BPA, and 3) provide evidence that variable dose levels of BPA act across different biological pathways [Vandenberg et al., 2009].

Further candidate gene methylation analysis in PND 22 liver tissue revealed alterations in DNA methylation profiles at the two imprinted genes *H19* and *Igf2*, but not in the non-imprinted genes *HsdBeta 11 Type 2*, *Ppar-alpha*, and *Ppar-gamma*. The famine of 1944-45 from the German imposed food embargo during World War 2 demonstrates that exposure to malnutrition during conception/early gestation resulted in the development of hypomethylation of the *IGF2* differentially methylated region (DMR) in whole blood of the offspring at ~ age 60 when compared to unexposed same-sex siblings [Heijmans et al., 2008]. Additionally, dietary exposure to 50 µg and 50 mg BPA/kg of diet in mice during embryonic days 0-12.5 displayed a decrease in embryonic *Igf2* DMR 1 methylation [Susiarjo et al., 2013]. This evidence along with our candidate gene analysis indicates the potential of early developmental insults to affect candidate gene CpG methylation, especially at imprinted loci. Continuing research should explore genome-

wide DNA methylation to discover novel regions of altered methylation and gene expression following perinatal BPA exposure in light of this evidence at candidate loci.

Due to BPA's ubiquitous existence in the environment and the ongoing debate about whether human internal BPA levels pose a health concern [Ginsberg et al., 2009; Volkel et al., 2002], it is of significance that animal studies capture human physiologically relevant exposure levels to determine BPA's impact on human health outcomes. In the present study, we aimed to achieve physiologically relevant levels by including a high (mg), medium (µg), and low (ng) dose of BPA in the maternal diet. Calafat *et al.* [2008] reported a range of urinary total BPA (free and conjugated) of 0.4 to 149 ng/mL representative of individuals 6 years of age or older (n = 2517) in subjects measured as a part of the 2003-2004 National Health and Nutrition Examination Survey (NHANES). Lang *et al.* [2008] reported urinary total BPA levels ranging from 3.34 to 4.48 ng/mL in individuals aged 18 years or older who have normal BMI (n = 469) from the 2003-2004 NHANES . Additionally, Padmanabhan *et al.* [2008] measured a range of 0.5 to 22.3 ng/mL of circulating free BPA (unconjugated) in maternal blood collected upon delivery.

We report PND 22 mouse liver BPA measurements ranging from below the limit of quantitation (LOQ) to 870 ng/g across all exposure groups comparable to human fetal liver measurements ranging from below LOQ to 96.8 ng/g. Ideally, liver BPA levels would be measured in fetal mouse tissues and compared to human fetal tissues; however, study design and current analytical requirements preclude this direct comparison. Nonetheless, mouse liver total and free BPA levels among A^{vy}/a offspring exposed to 50 mg BPA/kg diet in our study range from 9.46 to 870 and 2.68 to 390 ng/g, respectively, (**Table 3.4**) and are comparable to mouse circulating serum BPA concentrations recently reported in adult mice exposed to 100 mg BPA-

d_6/kg diet *ad libitum* for a 24 hour period [Sieli et al., 2011]. Sieli *et al.* [2011] show that in comparison to mice receiving a single oral bolus exposure of 20 mg BPA/kg body weight, animals fed BPA in the diet reach a maximum serum concentration of total and unconjugated (free) BPA at 6 hours of 802 and 18.8 ng/mL, respectively compared to 1 hour in the bolus group. Moreover, the observed serum concentrations following BPA administration in the diet are within the range of human exposure. Within the current study, we also observe a high degree of inter-individual variation in mouse liver BPA concentrations within a particular dose group as well as the controls, perhaps reflecting time and metabolism effects associated with recent feeding bouts and/or continued nursing of pups. Unlike single bolus ingestion or injection routes of exposure, dietary exposure through feed results in inherent inter-individual variability. It is important to note that we do not see a profound difference in mean or median mouse liver BPA concentrations among control and the two low dose groups; in fact, BPA is detected in some control animals despite housing in BPA-free caging and receiving BPA-free water. A limitation of this study is possible BPA cross-contamination via air given that animals were housed in a single room to minimize environmental heterogeneity that contributes to underlying epigenetic lability.

3.6 Conclusion

To our knowledge, this is the first study conducted using the viable yellow agouti epigenetic biosensor to analyze offspring phenotypic and epigenetic effects following multiple dose levels of either an environmental exposure or nutritional agent. Isogenic A^{vy} mice allow for reproducible experiments as seen here with the coat color shift towards yellow in the A^{vy}/a offspring exposed to the 50 mg BPA/kg diet [Dolinoy et al., 2007]. We took a candidate gene approach focusing on metastable epialleles unique to murine models and imprinted genes

associated with early life environmental perturbations, along with a global CCGG sequence assay. In order to conduct an unbiased epigenetic analysis, genome-wide methylation experiments must be applied in animal models. Epigenome-wide approaches will generate a template useful for the foundation and understanding of the full effect of BPA on the mouse epigenome. Concurrent studies are needed to assess BPA's effect on the human epigenome, and whether labile loci between the mouse and human display significant overlap. In understanding the epigenome as a whole, one must also consider other epigenetic mechanisms such as histone modifications and microRNA interference separately and in conjunction with each other, and their sensitivity to environmental disruptions. Recently, DNA methylation and histone modifications have been observed to act in concert with one another at the A^{vy} metastable epiallele [Dolinoy et al., 2010]. Increasing studies focusing on multiple epigenetic mechanisms will strengthen the understanding of physiologically induced alterations to the epigenome.

Table 3.1 PCR primers and sequences. Primers (5' to 3') and sequences to analyze for DNA methylation quantification via pyrosequencing for candidate genes. Y in sequence to analyze signifies a CpG site of interest.

Primer/Sequence to Analyze	
A^{vy} Assay	
Forward PCR	ATTTTTAGGAAAAGAGAGTAAGAAGTAAG
Reverse PCR	CTACAAAAACTCAAAAACTCA
Sequencing	TAGAATATAGGATGTTAG
Sequence to analyze	YGTGTTATTTTGTGAYGTGGYGTGAATGTGGGGGYGTGGTT
$Cabp^{IAP}$ Assay	
Forward PCR	ATTATTTTTTGATTGGTTGTAGTTTATGG
Reverse PCR	CACCAACATACAATTAACA
Sequencing	TAGAATATAGGATGTTAG
Sequence to analyze	YGTGTTATTTTGTGAYGTGGYGTGAATGTGGGGGYGTGGTT
Igf2 DMR Assay	
Forward PCR	TTTTTTAATATGATATTTGGAGATAGTT
Reverse PCR	CCACATAATTTAATTCACTAATAATTACTA
Sequencing	AATATGATATTTGGCGATAGTT
Sequence to analyze	YGYGGGAYGTTTGYGTAGAGGTTTGTTTGTTTTTTTGYGTGTTYGTYGGGGTYGT
H19 Assay	
Forward PCR	GGGGGGTTATAAATGTTATTAGGGGGGTAGG
Reverse PCR	AACCCCTAACCTCATAAAACCCATAACTATAAAATCA
Sequencing	GTGTAAAGATTAGGGTTGT
Sequence to analyze	GYGGTYAGTGAAGTTTYGTATATYG
HsdBeta 11 Type 2 Assay	
Forward PCR	GGGGTGTTAAGATAGAAGTGGA
Reverse PCR	AACAACAAACCCTAACACAACCAATC
Sequencing	AGTGGAGGAAAGTTTTA
Sequence to analyze	TYGTTTTYGTATYGTTTTAATTTYGTTTTTAGGGYG
Ppar-alpha Assay	
Forward PCR	Qiagen primer: PM00275758
Reverse PCR	Qiagen primer: PM00275758
Sequencing	Qiagen primer: PM00275758
Sequence to analyze	AGAGYGAGTTGGGGTAYGYGGT
Ppar-gamma Assay	
Forward PCR	TGGTGTGTATTTTATTGTAATTTTAAAAAG
Reverse PCR	ACTATCCTAACTAAAAACCAATTATAACT
Sequencing	TTTTGTTTTGGTAATTTTAATTAT
Sequence to analyze	TGTATAGTTTAYGTGTTTTTTATAGAATAGTGAATGTGTGGGTTATTGGYGTG

Table 3.2 Global DNA methylation. LUMA methylation levels in tail DNA among A^{vy}/a and *a/a* offspring

Exposure	N	Mean percent methylation (SD)	*P*-value (compared to control)
control	60	51.6 (17.9)	
mg	71	59.3 (15.2)	0.02
µg	52	60.3 (13.4)	0.007
ng	74	58.8 (9.8)	0.01

Table 3.3 Percent methylation summary. Methylation status of A^{vy} and $Cabp^{IAp}$ loci from tail DNA

Locus	N	Exposure	Mean percent methylation (SD) across CpG sites 6-9	Mean percent methylation (SD) site 6	Mean percent methylation (SD) site 7	Mean percent methylation (SD) site 8	Mean percent methylation (SD) site 9
A^{vy}	38	control	35.6 (26.4)	34.5 (26.4)	35.2 (23.2)	35.5 (27.2)	37.3 (30.3)
(A^{vy}/a offspring)	43	mg	24.3 (18.7)**	23.7 (18.9)**	24.1 (16.5)**	22.6 (18.8)**	26.7 (21.6)*
	32	µg	35.9 (27.5)	34.2 (27.8)	34.7 (23.7)	35.4 (27.7)	39.2 (31.5)
	48	ng	34.1 (25.2)	33.1 (25.5)	32.6 (21.9)	33.7 (25.4)	37.2 (28.8)
$Cabp^{IAP}$	82	control	83.1 (8.2)	84.6 (8.1)	81.5 (8.4)	78.5 (9.7)	88.0 (9.3)
(A^{vy}/a and a/a	85	mg	83.6 (6.4)	85.8 (6.4)	81.4 (6.9)	79.1 (7.1)	88.3 (7.7)
offspring)	67	µg	85.8 (5.6)**	87.5 (5.4)**	83.3 (6.6)	81.8 (6.5)**	90.6 (6.2)**
	107	ng	84.3 (6.0)	86.0 (5.8)	82.3 (7.2)	79.3 (7.6)**	89.6 (6.1)**

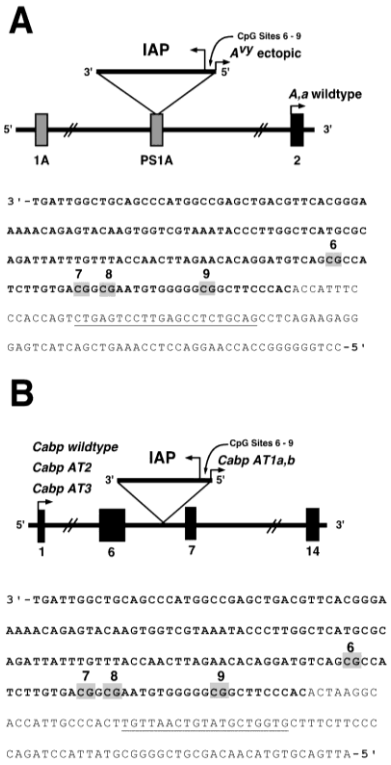
***indicates $P < 0.10$ compared to control exposure group. **indicates $P < 0.05$ compared to control group.**

Table 3.4 BPA liver concentrations (ng/g). Measurements from mouse PND 22 A^{vy}/a offspring tissue liver and human fetal liver tissue ranging from gestational ages 74 to 120

	Mice fed mg/kg diet (n = 9)	**Mice fed µg/kg diet (n = 10)**	**Mice fed ng/kg diet (n = 11)**	**Mice fed control diet (n = 10)**	**Human fetal (n = 51)**
Range Total BPA, ng/g	9.5-870	<LOQ-11.3	<LOQ-13	<LOQ-11.5	<LOQ-96.8
Mean (SD) free-BPA, ng/g	164 **(**132)	1.8 **(**3.5)	1.8 **(**2.9)	3.7 **(**2.8)	7.6 **(**12.2)
Mean (SD) conjugated-BPA, ng/g	278 **(**233)	0.3 **(**0.3)	1.0 **(**1.6)	0.6 **(**0.8)	3.2 **(**8.0)
Mean (SD) total BPA, ng/g	441 **(**338)	2.0 **(**3.5)	2.8 **(**4.5)	4.3 **(**3.5)	10.8 **(**18.5)
Median Total BPA, ng/g	472	0.6	0.3	4.2	3.4

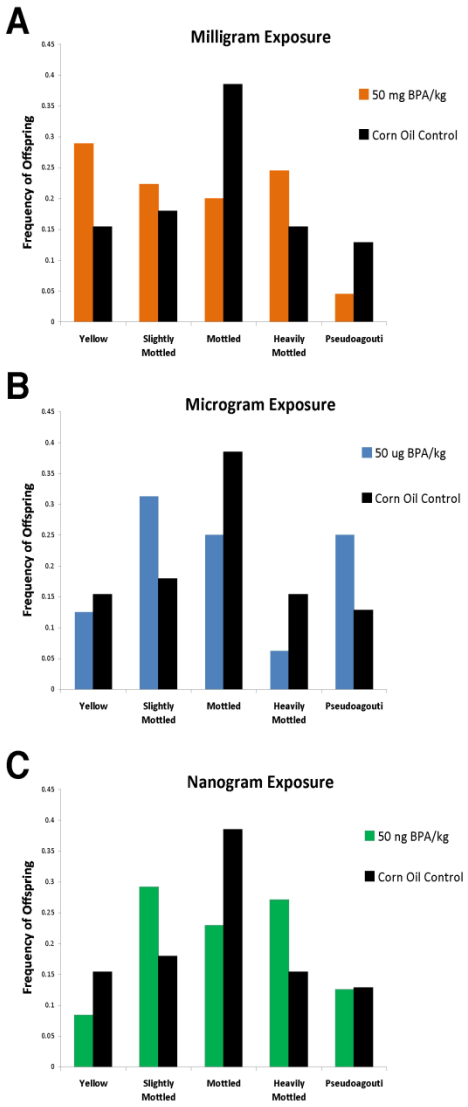
***LOQ = Limit of Quantitation (0.1 ng/g)**

Figure 3.1 A^{vy} and *Cabp* loci



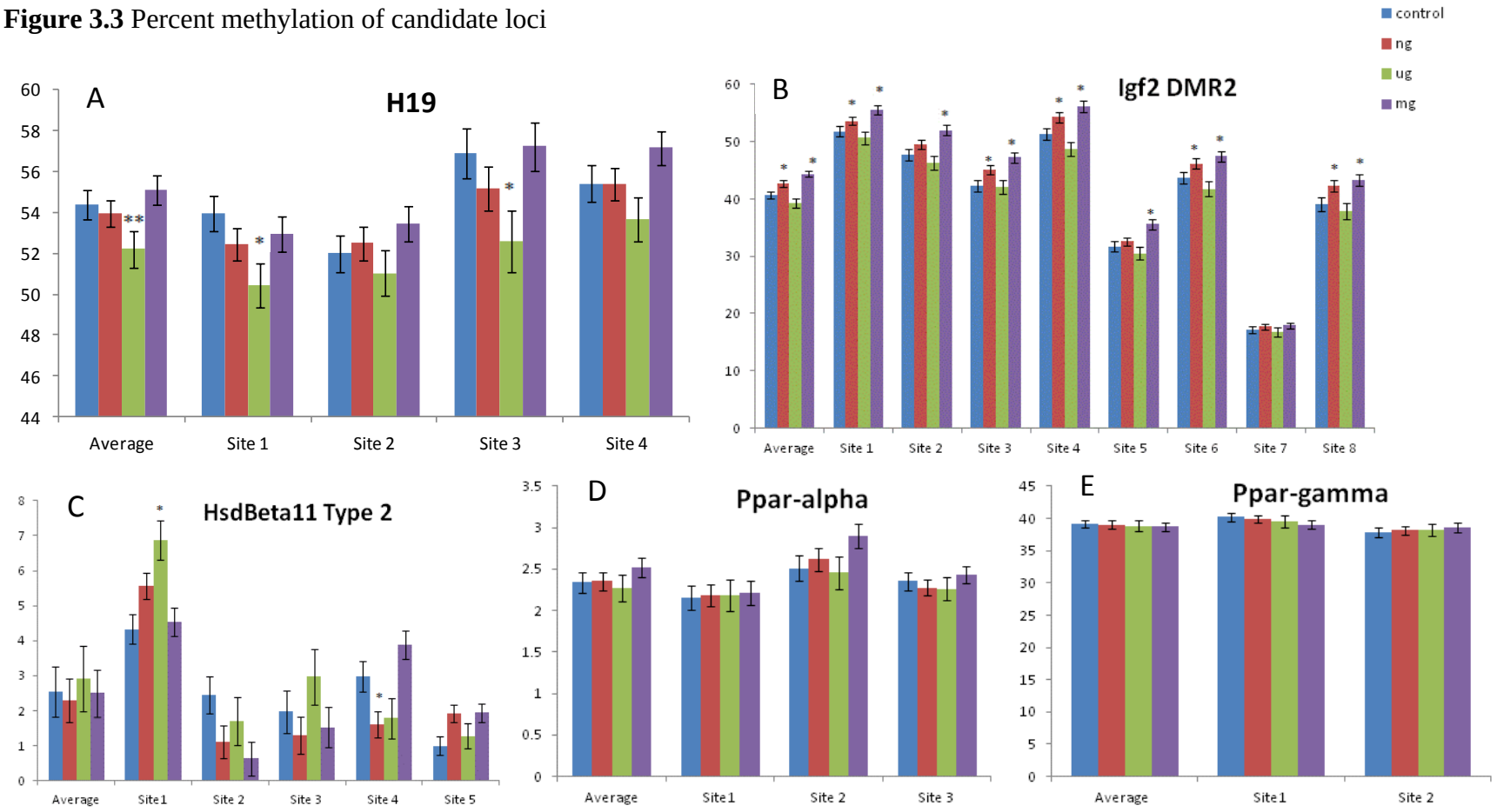
A) The A^{vy} allele contains a contra-oriented IAP insertion within pseudoexon 1A (PS1A) of the *Agouti* gene. A cryptic promoter (arrowhead labeled A^{vy} ectopic) drives constitutive ectopic *Agouti* expression. Transcription of the *Agouti* gene normally initiates from a developmentally regulated hair-cycle promoter in exon 2 (arrohwhead labeled A, a wildtype). The location of the bisulfite-converted genomic reverse primer for amplifying the A^{vy} IAP is underlined. **B)** The $Cabp^{IAP}$ allele contains a contra-oriented IAP insertion within intron 6 of the murine CDK5 activator binding protein (*Cabp*) gene, resulting in short aberrant transcripts originating from the 5'LTR of the IAP (arrowhead labeled *Cabp* AT1a,b). Aberrant transcripts also originate at the normal transcription start site (arrowhead labeled *Cabp* wildtype) and truncate 5' of the IAP insertion (*Cabp* AT2 and AT3). Normal *Cabp* transcription covers 14 exons, resulting in a 2kb transcript. Location of the bisulfite-converted genomic reverse primer for amplifying the $Cabp^{IAP}$ locus is underlined.

Figure 3.2 Coat color distribution



A) Coat color phenotype distribution among 50 mg/kg BPA exposed offspring (n=45) versus control offspring (n=38). 50 mg/kg BPA maternal intake demonstrates a shift in offspring coat color towards yellow (*P*=0.006). **B)** Coat color phenotype distribution among 50 µg/kg BPA offspring (n=32) versus control offspring. 50 µg/kg BPA maternal intake demonstrates a shift in coat color towards pseudoagouti (*P*=0.04). **C)** Coat color phenotype distribution among 50 ng/kg BPA offspring (n=48) versus control offspring. 50 ng/kg BPA maternal intake demonstrates a shift in offspring coat color towards heavily mottled (*P*=0.02).

Figure 3.3 Percent methylation of candidate loci



Average and CpG site-specific DNA methylation levels in **A)** *H19*, **B)** *Igf2*, **C)** *HsdBeta11 Type 2*, **D)** *Ppar-alpha*, and **E)** *Ppar-gamma* in PND 22 liver tissue of A^{vy}/a and *a/a* offspring. The y-axis represents percent methylation. *indicates $P<0.05$ compared to control exposure group.**indicates $P<0.10$ compared to control exposure group.

3.7 References

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CHAPTER 4

Novel epigenetic biomarkers mediating bisphenol A exposure and metabolic phenotypes

4.1 Abstract

There is compelling evidence that epigenetic modifications link developmental environmental insults to disease susceptibility in adulthood. Animal studies have linked perinatal bisphenol A (BPA) exposure to altered DNA methylation, but these studies have been limited to candidate gene and global methylation approaches. Utilizing an epigenome-wide discovery platform, this chapter elucidates epigenetic alterations in liver tissue from adult offspring (10 months) following perinatal BPA exposure at human physiologically relevant doses (50 ng, 50 μg, and 50 mg BPA/kg diet). Biological pathway analysis was run on the significant differentially methylated regions among females to determine which pathways were enriched, and whether they are involved in the metabolic or hyperactive phenotypes displayed in the BPA exposed offspring from the life-course phenotyping described in Chapter 2. Furthermore, utilizing the top enriched biological pathways, three candidate genes were chosen to assess DNA methylation as a mediating factor linking the association of perinatal BPA exposure to the metabolic phenotypes in female offspring. Of the significant differentially methylated probes, the biologically pathways that were enriched include insulin signaling, adipocytokine signaling, and tyrosine hydroxylase pathways. From these pathways, three candidate genes, *Insulin Receptor Substrate-2 (Irs-2), Janus Kinase-2 (Jak-2),* and *Tyrosine Hydroxylase (Th)* were chosen for a

mediational regression analysis. DNA methylation in two of the candidate genes, *Irs-2* and *Jak-2*, was linked as a mediator in the mechanistic pathways of BPA exposed females and the adiponectin and glucose phenotypes, respectively. Data generated from this study is crucial for deciphering the role of epigenetics in the pathogenesis of chronic disease and the development of novel epigenetic-based prevention and therapeutic strategies for complex human disease.

4.2 Introduction

The developmental origins of health and disease hypothesis (DOHaD) postulates that early nutritional and environmental exposures shape health outcomes throughout the life-course [Barker, 2004]. Bisphenol A (BPA), a chemical found in consumer products including beverage and food containers and thermal papers, is an environmental exposure that has been associated with disease development in adulthood after early-life exposure [Vandenberg et al., 2010]. BPA is an endocrine active compound that interferes with estrogen [Gould et al., 1998; Kuiper et al., 1998], androgen [Kruger et al., 2008], and thyroid function [Moriyama et al., 2002] as well as transcription factors like peroxisome x receptor (PXR) and aryl hydrocarbon receptor (AhR) [Kruger et al., 2008; Sui et al., 2012]. Additionally, BPA has the ability to impair the development of the central nervous system resulting in behavioral disorders [Braun et al., 2011; Wolstenholme et al., 2011]. Thus, BPA's influence on normal differentiation and maturation processes during early development, with health risk manifesting later in life, is of concern. When elucidating mechanisms involved in DOHaD, epigenetic pathways, such as DNA methylation, histone modifications and chromatin modeling, are a primary mechanism of interest [Gabory et al., 2011]. Of these epigenetic modifications, DNA methylation is the most widely studied and best characterized. DNA methylation is generally thought to be a stable mark

established early in development, but increasing evidence reveals that environmental and nutritional factors can drive sustained alterations to DNA methylation when encountered early in development. Recently, using the viable yellow agouti (A^{vy}) mouse model, our group has shown that maternal dietary exposure to three physiologically relevant levels of BPA (50 mg, µg, or ng BPA/kg diet) results in modifications to the A^{vy} and $Cabp^{IAP}$ metastable epialleles and the *Igf2*, and *H19* imprinted loci. Perinatal exposure to 50 mg BPA/kg diet resulted in decreased DNA methylation at the A^{vy} locus [Anderson et al., 2012; Dolinoy et al., 2007], while perinatal exposure to the lower doses, 50 ng and 50 µg BPA/kg diet, resulted in increased methylation at the $Cabp^{IAP}$ locus [Anderson et al., 2012]. Perinatal exposure to 50 µg BPA/kg diet resulted in decreased DNA methylation at *H19*, while perinatal exposure to 50 ng and 50 mg BPA/kg diet, resulted in increased methylation at the *Igf2* locus (**Figure 3.3B**).

Developmental exposures to BPA that have been associated with alterations in epigenetic modifications implicate the risk for chronic disease such as cancer, type 2 diabetes, and obesity as well as impaired brain development and behavior [Jirtle et al., 2007; Kundakovic et al., 2011]. For example, exposure to 10 µg BPA/kg BW/day in male rats during early development resulted in hypomethylation at the *phosphodiesterase type 4 variant 4* gene in prostate cancer cells during adulthood [Ho et al., 2006]. *In utero* exposure to 5 mg/kg body weight resulted in decreased methylation in *Hoxa10*, a gene involved in several cancers like endometrial carcinoma, in adult female mice [Bromer et al., 2010; Yoshida et al., 2006]. Two additional genes implicated in prostate carcinogenesis, *nucleosome binding protein-1* and *hippocalcin-like 1,* displayed hypo- and hypermethylation, respectively, in adult rats exposed to 10 µg BPA/kg body weight during the neonatal period [Tang et al., 2012].

Most studies aimed to investigate the effects on the epigenome following environmental and nutritional exposures have been limited by being candidate gene driven approaches or based on epigenetic techniques with limited genome coverage and sensitivity. Given that the main goal of DOHaD research is to understand the role that early environmental insults have on health and disease, deep sequencing and tiling array technologies are of most value in order to elucidate key regulatory pathways that are involved in the etiology of disease via epigenetic mechanisms. Further, following animals perinatally exposed across the life-course, helps identify a role for early exposure and later-in-life disease risk. Thus, by utilizing an epigenome-wide platform as a discovery tool in combination with life-course sophisticated in vivo phenotyping, we now examine regions of altered methylation (RAMs) as the potential mediators on the development of metabolic phenotypes in adulthood after perinatal BPA exposure.

4.3 Materials and Methods

4.3.1 Mouse Liver Samples and DNA isolation

The liver tissues utilized for this chapter include the BPA exposed and control non-agouti *a/a* wild-type offspring that were aged out to 10 months and underwent the metabolic phenotyping described in Chapters 1 and 2. For this study, 10 month liver DNA from a subset of the *a/a* offspring was analyzed for altered regions of methylation in murine promoter regions: 1) standard control diet (n=6 offspring; 3 male and 3 female); 2) 50 ng BPA/kg diet (n=6 offspring; 3 male and 3 female); 3) 50 µg BPA/kg diet (n=5 offspring; 2 male and 3 female); 4) 50 mg BPA/kg diet (n=6 offspring; 3 male and 3 female).

Total genomic DNA was isolated from 10 month liver tissue (n=23) using a standard phenol-chloroform extraction method. Briefly, about 15 mg of tissue was resuspended in 540 µl buffer ATL and homogenized for 20 seconds at 15 Hz (TissueLyser, Qiagen). The lysate was transferred to 60 µl of Proteinase K and incubated for overnight at 50□C. After overnight incubation 12 µl of RNase A was added to lysate and incubated for 10 minutes at 37□C. Samples were extracted twice with 600 µl phenol–chloroform–isoamyl and once with 600 µl chloroform. 50 µL of 3M sodium acetate was added to aqueous phase and precipitated with 1 mL ice-cold 100% ethanol. For additional precipitation, 1 mL of ice-cold75% ethanol was added to pellet twice. The pellet was air dried and resuspended with 100 µl Tris-EDTA buffer and incubated for 2 hours at 60□C with frequent mixing.

4.3.2 DNA Fragmentation

Genomic DNA was sheared to fragment sizes between 200 and 1000 bp. Briefly, 17 µg of DNA was divided into equal volumes into 2 separate PCR tubes (8.5 ug DNA each). DNA was sonicated with a total process time of 15 minutes with 15 second on and 30 seconds off cycles at an amplitude of 18 (Episonic 1100 series, Farmingdale, NY) in 8-20□C water (monitored and cooled every 5 minutes of process time). Fragment sizes were confirmed by gel electrophoresis with ~1 µg DNA.

4.3.3 Enrichment of Methylated DNA

Methylated regions of fragmented DNA were enriched by methyl-CpG binding domain-based capture (MBD-Cap) using the Methylated DNA Enrichment Kit (Epimark, New England BioLabs, Ipswich, MA). Methylated DNA binds to the methyl CpG binding domain of human

MBD2 protein, which is coupled to paramagnetic protein A beads. First, the MBD2-Fc and beads were combined, incubated by rotation for 15 minutes at room temperature, and washed twice. The fragmented DNA was then added to the MBD2-Fc/bead mixture and incubated by rotation for 20 minutes at room temperature and washed three times to discard unbound DNA. The captured methylated CpG DNA was eluted with 100 µl of DNase-free water and 15 minutes of incubation at 65□C.

4.3.4 Whole Genome Amplification, and Enrichment Quality Assessment via qPCR

DNA enriched for CpG methylation underwent whole genome amplification (WGA) according to the manufacturer's instructions using GenomePlex□ Complete Whole Genome Amplification Kit (Sigma, St. Louis, MO). Validation and quality assurance of the enriched fraction was performed using a positive methylated control (*Xist)* and a negative unmethylated control (*H19*) loci via qPCR. Briefly, 60 ng of the genomic sonicated DNA, non-captured fraction from enrichment, and WGA enriched fraction were prepared as 1:10 serial dilutions. qPCR components were prepared using SYBR Green master mix (Qiagen Inc., Valencia, CA), forward primer (0.25 pmol), and reverse primer (0.25 pmol). The qPCR was performed using the following parameters: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 62□C for 30 seconds, elongation at 72□C for 10 seconds, repeated for 39 cycles starting at the second denaturation step. All samples were run in duplicate. The cycles to threshold were averaged across duplicates and used to quantitate enrichment of the IP fraction compared to the input and non-captured fraction by taking the difference of the cycles to threshold.

4.3.5 Hybridization and Array Scanning

Experimental (IP) and control (input) samples were labeled with Cy5 and Cy3, respectively with the NimbleGen Dual-Color DNA Labeling Kit (Roche, Indianapolis, IN) following the NimbleGen Arrays User Guide (NimbleGen Arrays User Guide *DNA Methylation Arrays,* Version 7.2). IP and input samples were pooled and co-hybridized to Roche NimbleGen Mouse DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Arrays for a period of 16 to 20 hours. After the hybridization period was complete, arrays were washed and scanned using 2-µm, high-resolution NimbleGen MS 200 Microarray Scanner (Dr. Thomas Glover, Department of Human Genetics, University of Michigan).

4.3.6 Bioinformatics Pipeline

All arrays were uploaded to NimbleGen DEVA Software (version 2.3). An alignment grid was laid over each subarray image in order to extract the location of each feature as well as to generate the raw signal intensities from the IP and input samples. The appropriate normalization procedure was determined by visualizing the distribution of each subarray. The normalization process consisted of two steps: 1) the log2 ratio of the input and IP channels were calculated; 2) the log2 ratios were corrected by subtracting the Tukey's biweight estimate of the median. The Tukey's biweight estimate is determined by a weight given to each data using a bi-square weight function. The weights assigned are greater for data values near the median of the data cluster and decrease for data away from the median, so outliers have a minimal effect on the estimate [Adriaens et al., 2012]. The following statistical analyses were performed with R Statistical Software (version 2.10.1). An empirical Bayesian T-test was calculated to assess the differences of the normalized log2 ratios using dichotomous comparisons of BPA exposure

groups (50 ng, 50 µg, or 50 mg BPA/kg diet) versus the control group. Analyses were done by pooling sexes as well as by stratifying by sex. Significant differentially methylated genes were chosen based on adjusted *P*-values <0.001.

4.3.7 Pathway Analysis

The top hit genes with adjusted *P*-values <0.001 were read into Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.7) for identification of key biological pathways involved among the most differentially methylated genes. For this dissertation, candidate genes were chosen from enriched biological pathways that may be involved the metabolic or hyperactive phenotypes displayed in the BPA exposed offspring described in Chapter 2. The pathway analysis was limited to females since the metabolic and hormone phenotypes were exhibited strictly in female offspring. The three candidate genes chosen from the enriched biological pathways are *Insulin Receptor Substrate-2* (*Irs-2*), *Janus Kinas-2* (*Jak-2*), and *Tyrosine Hydroxalase* (*Th*).

4.3.8 Mediational Regression Analysis

Mediational regression analysis was conducted in a 4-step process to determine whether DNA methylation is a mediator of BPA exposure and metabolic phenotypes. First, a mixed effects regression analysis was performed to identify significant associations among BPA exposure (50 ng, 50 µg, or 50 mg BPA/kg diet) and the metabolic phenotypes (model 1) described in Chapter 2 (spontaneous activity, energy expenditure, body composition, and hormones in 9 and 10 month old offspring) in the female exposed offspring versus the control female offspring that were used for epigenome-wide analysis as described above. Second,

analyses from the empirical Bayesian T-test, described in the previous section, were utilized to determine the significance of the associations among BPA exposure and DNA methylation (model 2) in each candidate gene among the BPA exposed groups compared to the control group. Third, a mixed effects regression was performed to determine the associations of DNA methylation in each candidate gene and the metabolic phenotypes (model 3). Finally, given that all three previous analyses (models 1, 2, and 3) resulted in a significant relationship, a final mixed effects regression model measuring BPA exposure and its association on the metabolic phenotypes adjusting for DNA methylation was performed (model 4). Methylation was considered a mediator if the difference estimate resulted in a 10% attenuation in model 4 (adjusting for methylation), compared to the model 1 (without adjusting for methylation). Due to the limited sample size, results were considered significant at $P<0.10$. All statistical analyses were completed using SAS software version 9.2 (Cary, NC).

4.4 Results

4.4.1 Identification of Differentially Methylated Regions Across BPA Exposed Offspring

The top and bottom 25% most differentially methylated probes indicate that there are clusters of genes presenting with increased and decreased methylation across all exposure groups compared to the controls (**Figure 4.1A**). Additionally, there are clusters of genes acting in a non-monotonic fashion across exposure groups (**Figure 4.1B**).

Among the 713,168 probes, about 90,000 (10%) unique probes exhibited differential methylation by BPA exposure given the adjusted p-value cut-off of 0.001 (**Figure 4.2**). Among ng exposed offspring, 0.9% of the loci were identified as differentially methylated compared to

controls (**Figure 4.2**). Among µg exposed offspring, 1.2% of the loci were identified as differentially methylated compared to controls (**Figure 4.2**). Among mg exposed offspring, 2.5% of the loci were identified as differentially methylated compared to controls (**Figure 4.2**).

4.4.2 Biological Pathways Involved in Differentially Methylated Regions

The pathways that were enriched in female BPA exposed offspring include the insulin signaling, adipocytokine signaling, and tyrosine metabolism pathways. The three candidate genes chosen from these pathways were *Insulin Receptor Substrate-2* (*Irs-2*), *Janus Kinase-2* (*Jak-2),* and *Tyrosine Hydroxalase* (*Th)* (**Table 4.1**). Additional enriched pathways that may be of interest to this project include, but are not limited to, Jak-STAT signaling pathway, MAPK signaling pathway, and pathways in cancer, for example (**Table 4.1)**

4.4.3 DNA Methylation Mediating BPA Exposure and Metabolic Phenotypes

Due to the small sample size (n=3 female per exposure group), there were limited associations when performing the regression analyses of BPA exposure predicting the metabolic phenotypes (without methylation adjustment; model 1). The trend and the differences of model 1, however, were similar to the initial mixed effects regression analysis performed in Chapter 2 on the full sample sizes of each exposure group (**Tables 4.2-4.12**).

After modeling adiponectin as the outcome, the differences from the control group were attenuated 10% in all exposure groups when adjusting for methylation at *Irs-2*. In model 1, the mg exposed female offspring displayed a significant increase in adiponectin levels ($P<0.0001$). The difference from the controls was attenuated (63%) after controlling for methylation at *Irs-2* in model 4 (**Table 4.9**), indicating that methylation status at *Irs-2* is a mediator of BPA exposure

and adiponectin levels (*P*=0.26). Additionally, in model 1, the µg exposed females displayed a significant increase in adiponectin levels (*P*=0.001). The difference from the controls was attenuated (33%) after controlling for methylation at *Irs-2* in model 4 (*P*=0.02) (**Table 4.9**). Finally, in model 1, the ng exposed females displayed a significant increase in adiponectin levels (*P*=0.0002). Again, the difference from the controls was attenuated (15%) after controlling for methylation at *Irs-2* in model 4 (*P*=0.0007) (**Table 4.9**).

After modeling baseline glucose as the outcome, the differences from the control group were attenuated <10% in all exposure groups when adjusting for methylation at *Jak-2*. In model 1, the mg exposed female offspring displayed an increase in adiponectin levels (*P*=0.34). The difference from the controls was attenuated (24%) after controlling for methylation at *Jak-2* in model 4 (**Table 4.11**), indicating that methylation status at *Jak-2* is a potential mediator of BPA exposure and adiponectin levels (*P*=0.51). Additionally, in model 1, the ∓g exposed females displayed an increase in adiponectin levels (*P*=0.68). The difference from the controls was attenuated (52%) after controlling for methylation at *Jak-2* in model 4 (*P*=0.86) (**Table 4.11**). Finally, in model 1, the ng exposed females displayed a marginally significant increase in adiponectin levels (*P*=0.18). The difference from the controls was attenuated (59%) after controlling for methylation at *Jak-2* in model 4 (*P*=0.71) (**Table 4.11**).

Methylation did not result in mediation after performing mediational regression analysis adjusting for DNA methylation at *Th* (**Tables 4.2-4.12**).

4.5 Discussion

Previous work as well as epigenetic analysis done in this dissertation has shown that physiologically relevant level of BPA exposure during early development results in altered levels

of methylation in candidate gene and global methylation assays [Anderson et al., 2012; Dolinoy et al., 2007; Ho et al., 2006; Susiarjo et al., 2013]. In this chapter we extend beyond the traditional candidate and global methylation approaches by taking on an epigenome-wide platform. A constellation of genomic loci in mouse promoter regions following perinatal BPA exposure were discovered. The discovery of novel regions of altered methylation can be utilized for the identification of genes involved in specific disease pathways that develop after exposure to an environmental insult.

The biological pathway analysis revealed an enrichment of genes involved in hormone regulation, like insulin and leptin, as well as in neural signaling pathways (**Table 4.1**). These pathways support data from our metabolic phenotyping data in Chapter 2, as well as other previously reported data on developmental exposure to BPA and resulting adult phenotypes [Ben-Jonathan et al., 2009; Mizuo et al., 2004; Zhou et al., 2011]. By utilizing biological pathway annotation tools, researchers that employ epigenome-wide platforms have the opportunity to identify genomic loci that are differentially methylated in a specific disease pathway. For example, Sabunciyan et al. examined DNA methylation of the prefrontal cortex from deceased individuals known to have major depressive disorder (MDD). Biological pathway analysis was run on the top hit differentially methylated regions to determine which genes may be epigenetic susceptible in the progression of MDD [Sabunciyan et al., 2012]. Additionally, identification of novel epigenomic biomarkers was discovered in patients with normal, premalignant, and oral cavity squamous cell carcinoma tumor tissue using an epigenome-wide platform [Guerrero-Preston et al., 2011]. The differentially methylated genes were found to be associated with the pathways closely related to oncogenic transformation following pathway enrichment. Thus, pathway analyses provides a means to understand the biological meaning

behind a large set of differentially methylated genes generated from high density epigenome-wide platforms.

In addition to identifying new differentially methylated genes by BPA exposure, we were able to combine genome-wide methylation data with previous life-course phenotyping to perform a meditational regression analysis to assess whether DNA methylation. The mediational regression analysis revealed two genes, *Irs-2 and Jak-2*, involved in hormone signaling as potential mediators of BPA exposure and the metabolic phenotypes including adiponectin and baseline glucose levels, respectively. Characterization of epigenetic biomarkers indicative of developmental BPA exposure and predictive of disease development will result in unique opportunities to develop disease prevention strategies and therapeutic modifications of these differentially methylated regions acting as mediators in individuals who may be predisposed to disease development or in individuals with existing disease in order to facilitate reversal of disease progression. Due to the plasticity of the epigenome [Waterland et al., 2003] such strategies may include dietary supplementation or pharmaceutical intervention [Dolinoy et al., 2007].

Future work for this Chapter includes conducting a technical and biological validation of the candidate genes using pyrosequencing technology, which will increase our statistical power when computing the mediational regression analysis, as all animals with *in vivo* life course phenotyping can be concluded. A limitation of epigenome-wide analyses is cost, and as such, only a subset of animals (23 of 79) was run on the NimbleGen array platform.

4.6 Conclusion

Over the past decade, epigenetic technologies have evolved from once traditional methods using restriction enzymes or candidate genes to modern technologies allowing for unbiased epigenome-wide investigation across tissues and species, yet most studies aimed to investigate the effects on the epigenome following environmental and nutritional exposures have been limited by being candidate gene or global driven approaches. Since the main goal of DOHaD research is to understand the role that early environmental insults have on health and disease, deep sequencing and tiling array technologies are of most value in order to elucidate key regulatory pathways that are involved in the etiology of disease via epigenetic mechanisms. Furthermore, there is now evidence that DNA methylation may act in concert with other epigenetic mechanisms such as histone modifications and chromatin remodeling complexes and should also be considered when evaluating the epigenome [Dolinoy et al., 2010].

Table 4.1 Biological pathway analysis. DAVID pathway analysis reveals insulin response, adipoctyokine signaling, and tyrosine metabolism pathways in BPA exposed female offspring

Pathway	Number of Genes	*P*-value
Pathways in cancer	305	1.3E-05
MAPK signaling	250	1.7E-03
Wnt signaling	144	8.8E-06
Jak-STAT signaling	142	1.9E-03
Melanoma	94	9.3E-03
Adipocytokine signaling	62	9.2E-02
Insulin signaling	124	8.4E-02
Purine metabolism	142	4.0E-02
Cell cycle	116	5.9E-02
Long term depression	67	6.0E-02
Neuro-active ligand receptor	234	1.2E-02
Tyrosine metabolism	37	4.2E-02
Basal cell carcinoma	54	6.2E-03
Gap Junction	81	1.6E-02

Table 4.2 Mediational regression analysis (Oxygen Consumption). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating oxygen consumption (ml/kg/hr) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean VO (se)	Overall *P*-value	Mean VO difference (se) vs control	*P*-value	Sample Size
Oxygen Consumption (VO)	Full Sample Size (None)			0.02			
		mg	3901 (160)		152 (219)	0.48	9
		ug	3473 (151)		-275 (212)	0.2	10
		ng	4525 (150)		774 (212)	0.003	10
		control	3749 (151)		-		10
	None (model 1)			0.26			
		mg	4471 (386)		924 (500)	0.07	3
		ug	3609 (318)		61 (450)	0.89	3
		ng	3933 (386)		385 (500)	0.44	3
		control	3547 (318)		-		3
	Insulin Receptor Substrate-2 (model 4)			0.006			
		mg	2051 (1057)		5409 (1589)	0.001	3
		ug	5635 (509)		1825 (833)	0.03	3
		ng	7111 (660)		349 (839)	0.68	3
		control	7460 (721)		-		3
	Janus Kinase-2 (model 4)			0.23			
		mg	4427 (423)		1037 (553)	0.07	3
		ug	3559 (349)		169 (497)	0.73	3
		ng	4146 (450)		756 (610)	0.22	3
		control	3389 (366)		-		3
	Tyrosine Hydroxalase (model 4)			0.14			
		mg	4593 (296)		1045 (380)	0.01	3
		ug	3603 (238)		55 (336)	0.87	3
		ng	3858 (292)		310 (376)	0.41	3
		control	3548 (238)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.3 Mediational regression analysis (Carbon Dioxide Production). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating carbon dioxide production (ml/kg/hr) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean VCO (se)	Overall *P*-value	Mean VCO difference (se) vs control	*P*-value	Sample Size
Carbon Dioxide Production	Full Sample Size (None)			0.59			
(VCO)		mg	3584 (182)		320 (250)	0.2	9
		ug	3692 (168)		428 (239)	0.08	10
		ng	3736(166)		472 (239)	0.05	10
		control	3263 (171)		-		10
	None			0.84			
	(model 1)	mg	3843 (356)		278 (462)	0.55	3
		ug	3743 (294)		178 (415)	0.67	3
		ng	3440 (356)		-124 (462)	0.79	3
		control	3564 (294)		-		3
	Insulin Receptor Substrate-2			0.02			
	(model 4)	mg	493 (1149)		-5088 (1613)	0.00	3
		ug	4066 (766)		-1515 (1131)	0.19	3
		ng	4386 (953)		-1195 (1225)	0.33	3
		control	5581 (863)		-		3
	Janus Kinase-2			0.84			
	(model 4)	mg	3797 (362)		399 (474)	0.40	3
		ug	3690 (299)		292 (426)	0.50	3
		ng	3669 (387)		270 (527)	0.61	3
		control	3398 (315)		-		3
	Tyrosine Hydroxalase			0.95			
	(model 4)	mg	3682 (339)		117 (434)	0.79	3
		ug	3752 (272)		186 (384)	0.63	3
		ng	3556 (334)		-8.6 (431)	0.98	3
		control	3565 (271)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.4 Mediational regression analysis (Ambulatory Activity). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating ambulatory activity (counts/hr) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean activity (se)	Overall *P*-value	Mean activity difference (se) vs control	*P*-value	Sample Size
Ambulatory Activity	Full Sample Size (None)			0.07			
		mg	1981 (142)		610 (195)	0.002	9
		ug	1868 (133)		497 (189)	0.009	10
		ng	1739 (131)		367 (187)	0.05	10
		control	1371 (134)				10
	None (model 1)			0.72			
		mg	1981 (254)		377 (335)	0.27	3
		ug	1715 (219)		111 (309)	0.72	3
		ng	1792 (254)		187 (335)	0.58	3
		control	1604 (218)				3
	Insulin Receptor Substrate-2 (model 4)			0.14			
		mg	2956 (528)		1939 (821)	0.02	3
		ug	1622 (198)		604 (364)	0.10	3
		ng	1502 (262)		484 (328)	0.15	3
		control	1017 (346)				3
	Janus Kinase-2 (model 4)			0.8			
		mg	1988 (280)		363 (376)	0.34	3
		ug	1721 (240)		97 (345)	0.78	3
		ng	1765 (331)		142 (478)	0.77	3
		control	1624 (269)				3
	Tyrosine Hydroxalase (model 4)			0.61			
		mg	1846 (178)		241 (237)	0.31	3
		ug	1721 (156)		116 (221)	0.60	3
		ng	1881 (176)		276 (236)	0.24	3
		control	1604 (156)				3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.5 Mediational regression analysis (Horizontal Activity). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating horizontal activity (counts/hr) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean activity (se)	Overall *P*-value	Mean activity difference (se) vs control	*P*-value	Sample Size
Horizontal Activity	Full Sample Size (None)			0.02			
		mg	3446 (180)		856 (251)	0.007	9
		ug	3241 (167)		651 (241)	0.007	10
		ng	3067(166)		476 (241)	0.05	10
		control	2591 (174)		-		10
	None (model 1)			0.75			
		mg	3320 (362)		499 (475)	0.30	3
		ug	3052 (308)		231 (436)	0.60	3
		ng	3165 (362)		344 (475)	0.47	3
		control	2821 (308)		-		3
	Insulin Receptor Substrate-2 (model 4)			0.21			
		mg	4577 (760)		2512 (1180)	0.04	3
		ug	2932 (286)		867 (525)	0.10	3
		ng	2796 (381)		731 (478)	0.13	3
		control	2065 (498)		-		3
	Janus Kinase-2 (model 4)			0.9			
		mg	3381 (475)		351 (630)	0.58	3
		ug	3119 (398)		88 (572)	0.88	3
		ng	2878 (538)		-152 (759)	0.84	3
		control	3031 (436)		-		3
	Tyrosine Hydroxalase (model 4)			0.51			
		mg	3128 (244)		307 (322)	0.34	3
		ug	3061 (211)		240 (298)	0.42	3
		ng	3290 (240)		469 (319)	0.15	3
		control	2820 (210)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.6 Mediational regression analysis (Vertical Activity). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating vertical activity (counts/hr) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean activity (se)	Overall *P*-value	Mean activity difference (se) vs control	*P*-value	Sample Size
Vertical	Full Sample Size (None)			0.03			
Activity		mg	1379 (125)		506 (188)	0.007	9
		ug	1501 (127)		628 (181)	0.0005	10
		ng	1201 (135)		328 (180)	0.07	10
		control	873 (129)		-		10
	None			0.84			
	(model 1)	mg	1164 (407)		124 (529)	0.81	3
		ug	1470 (338)		430 (477)	0.37	3
		ng	1186 (407)		146 (529)	0.78	3
		control	1039 (338)		-		3
	Insulin Receptor Substrate-2			0.23			
	(model 4)	mg	2458 (762)		2194 (1173)	0.07	3
		ug	1346 (301)		1083 (538)	0.05	3
		ng	814 (403)		551 (507)	0.28	3
		control	264 (501)		-		3
	Janus Kinase-2			0.86			
	(model 4)	mg	1171 (430)		105 (566)	0.85	3
		ug	1478 (357)		412 (511)	0.42	3
		ng	1151 (472)		84 (654)	0.90	3
		control	1066 (383)		-		3
	Tyrosine Hydroxalase						
	(model 4)	mg	1029 (332)	0.63	-10.9 (426)	0.98	3
		ug	1477 (267)		437 (378)	0.25	3
		ng	1283 (326)		242 (422)	0.57	3
		control	1040 (267)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.7 Mediational regression analysis (Body Weight). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating body weight (g) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean BW (se)	Overall *P*-value	Mean BW difference (se) vs control	*P*-value	Sample Size
Body Weight	Full Sample Size (None)			0.59			
(BW)		mg	28.9 (1.5)		-3.1 (2)	0.1	9
		ug	30.3 (1.4)		-1.8 (1.9)	0.4	10
		ng	28.4 (1.3)		-3.7 (1.9)	0.06	10
		control	32.1 (1.4)		-		10
	None			0.83			
	(model 1)	mg	29.8 (3.1)		0.27 (4.3)	0.95	3
		ug	31.4 (3.1)		1.9 (4.3)	0.68	3
		ng	27.4 (3.1)		-2.1 (4.1)	0.63	3
		control	29.5 (3.1)		-		3
	Insulin Receptor Substrate-2			0.64			
	(model 4)	mg	21.5 (8.3)		-13 (13.1)	0.35	3
		ug	32.2 (3.1)		-2.3 (5.8)	0.7	3
		ng	29.9 (3.9)		-4.5 (4.8)	0.38	3
		control	34.5 (5.5)		-		3
	Janus Kinase-2			0.45			
	(model 4)	mg	30.5 (2.9)		-1.7 (4.3)	0.70	3
		ug	32.2 (2.9)		0.06 (4.2)	0.99	3
		ng	23.2 (4)		-8.9 (6.2)	0.19	3
		control	32.2 (3.4)		-		3
	Tyrosine Hydroxalase			0.61			
	(model 4)	mg	31.3 (3.1)		1.8 (4.2)	0.68	3
		ug	31.3 (2.9)		1.8 (4.1)	0.68	3
		ng	25.9 (3.1)		-3.6 (4.2)	0.42	3
		control	29.5 (2.9)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.8 Mediational regression analysis (Body Fat). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating body fat (g) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean BF (se)	Overall *P*-value	Mean BF difference (se) vs control	*P*-value	Sample Size
Body Fat	Full Sample Size (None)			0.76			
(BF)		mg	4.5 (.73)		-1.7 (1)	0.1	9
		ug	5.2 (.68)		-.93 (.96)	0.33	10
		ng	4.4 (.67)		-1.7 (.96)	0.08	10
		control	6.2 (.68)		-		10
	None			0.73			
	(model 1)	mg	5.2 (1.8)		0.87 (2.5)	0.74	3
		ug	6.6 (1.8)		2.2 (2.5)	0.4	3
		ng	3.9 (1.8)		-4.7 (2.5)	0.86	3
		control	4.3 (1.8)		-		3
	Insulin Receptor Substrate-2			0.42			
	(model 4)	mg	1.3 (4.5)		-7.5 (7.1)	0.22	3
		ug	7.1 (1.7)		-1.03 (3.2)	0.75	3
		ng	5.8 (2.1)		-2.3 (2.6)	0.40	3
		control	8.2 (3)		-		3
	Janus Kinase-2			0.31			
	(model 4)	mg	5.7 (1.6)		-0.45 (2.4)	0.85	3
		ug	7.1 (1.6)		1.0 (2.4)	0.68	3
		ng	1.1 (2.3)		-5.1 (3.4)	0.19	3
		control	6.1 (1.9)		-		3
	Tyrosine Hydroxalase			0.39			
	(model 4)	mg	6.3 (1.6)		2 (2.3)	0.40	3
		ug	6.5 (1.5)		2.1 (2.2)	0.35	3
		ng	2.7 (1.6)		-1.5 (2.2)	0.51	3
		control	4.3 (1.5)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.9 Mediational regression analysis (Adiponectin). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating adiponectin (∓g/ml) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean adiponectin (se)	Overall *P*-value	Mean adiponectin difference (se) vs control	*P*-value	Sample Size
Adiponectin	Full Sample Size (None)			0.08			
		mg	47.2 (7.5)		15.9 (10.2)	0.13	9
		ug	35.2 (6.5)		4.1 (9.4)	0.67	10
		ng	57.3 (7.5)		26.2 (10.2)	0.02	10
		control	31.1 (6.9)		-		10
	None			0.0002			
	(model 1)	mg	45.3 (2.3)		27.7 (3.3)	<.0001	3
		ug	34.2 (2.3)		16.6 (3.3)	0.001	3
		ng	38.6 (2.3)		21.0 (3.3)	0.0002	3
		control	17.6 (2.3)		-		3
	Insulin Receptor Substrate-2			0.0024			
	(model 4)	mg	34.3 (5.3)		10.1 (8.3)*	0.2632	3
		ug	35.3 (2.0)		11.1 (3.7)*	0.0192	3
		ng	42.0 (2.4)		17.8 (3.1)*	0.0007	3
		control	24.2 (3.5)		-		3
	Janus Kinase-2			0.0003			
	(model 4)	mg	46 (2)		25.7 (3)	<.0001	3
		ug	35.1 (2)		14.9 (2.9)	0.0015	3
		ng	34.5 (2.8)		14.3 (4.3)	0.0127	3
		control	20.2 (2.4)		-		3
	Tyrosine Hydroxalase			0.0003			
	(model 4)	mg	44 (2.3)		26.3 (3.2)	<.0001	3
		ug	34.3 (2.2)		16.6 (3.1)	0.001	3
		ng	39.8 (2.3)		22.2 (3.2)	0.0002	3
		control	17.6 (2.2)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.10 Mediational regression analysis (Leptin). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating leptin (ng/ml) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean leptin (se)	Overall *P*-value	Mean leptin difference (se) vs control	*P*-value	Sample Size
Leptin	Full Sample Size (None)			0.38			
		mg	11.2 (3.1)		-6.7 (4.3)	0.14	9
		ug	13.6 (2.7)		-4.4 (4)	0.29	10
		ng	17.3 (3.2)		-.65 (4.4)	0.88	10
		control	18 (3)		-		10
	None			0.99			
	(model 1)	mg	14.5 (5.6)		-0.11 (8)	0.99	3
		ug	16.5 (5.6)		1.7 (7.8)	0.83	3
		ng	16.6 (6.9)		1.9 (8.9)	0.83	3
		control	14.7 (5.6)		-		3
	Insulin Receptor Substrate-2			0.24			
	(model 4)	mg	3.2 (14.7)		-23.1 (23.7)	0.27	3
		ug	18.5 (5.6)		7.3 (10.4)	0.51	3
		ng	23.5 (8.5)		2.3 (9.1)	0.80	3
		control	25.8 (10.1)		-		3
	Janus Kinase-2			0.99			
	(model 4)	mg	14.8 (6.1)		-0.73 (9.1)	0.94	3
		ug	16.7 (6.2)		1.2 (9)	0.90	3
		ng	14.9 (11.4)		-0.55 (15.6)	0.97	3
		control	15.5 (7.2)		-		3
	Tyrosine Hydroxalase			0.87			
	(model 4)	mg	18.3 (5.1)		3.6 (6.9)	0.62	3
		ug	16.2 (4.8)		1.6 (6.7)	0.82	3
		ng	11.3 (6.4)		-3.3 (7.9)	0.69	3
		control	14.7 (4.7)		-		3

***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.11 Mediational regression analysis (Baseline Glucose). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating baseline glucose (mg/dl) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean glucose (se)	Overall *P*-value	Mean glucose difference (se) vs control	*P*-value	Sample Size
Baseline Glucose	Full Sample Size (None)			0.1			
		mg	97.2 (6.7)		19.7 (9.3)	0.05	9
		ug	112.6 (5.9)		4.4 (8.8)	0.62	10
		ng	116.6 (6.5)		0.37 (9.2)	0.97	10
		control	117 (6.5)		-		10
	None			0.5			
	(model 1)	**mg**	115.6 (9.4)		-13.3 (13.3)	0.34	3
		ug	123.3 (9.4)		-5.7 (13.3)	0.68	3
		ng	109.7 (9.4)		-19.3 (13.3)	0.18	3
		control	129 (9.4)		-		3
	Insulin Receptor Substrate-2			0.62			
	(model 4)	**mg**	113.1 (28)		-17.4 (43.4)	0.7	
		ug	123.6 (10.3)		-7 (19.2)	0.72	
		ng	110.5 (13)		-20.1 (16)	0.25	
		control	130.5 (18)		-		
	Janus Kinase-2			0.9			
	(model 4)	**mg**	114.5 (9.8)		-10.1 (14.4)*	0.51	3
		ug	121.9 (9.9)		-2.7 (14.3)*	0.86	3
		ng	116.6 (13.7)		-8 (21)*	0.71	3
		control	124.6 (11.5)		-		3
	Tyrosine Hydroxalase			0.46			
	(model 4)	mg	119 (10)		-9.8 (13.8)	0.50	3
		ug	123 (9.4)		-5.8 (13.3)	0.67	3
		ng	106 (10)		-22.6 (13.8)	0.14	3
		control	129 (9.4)		-		3

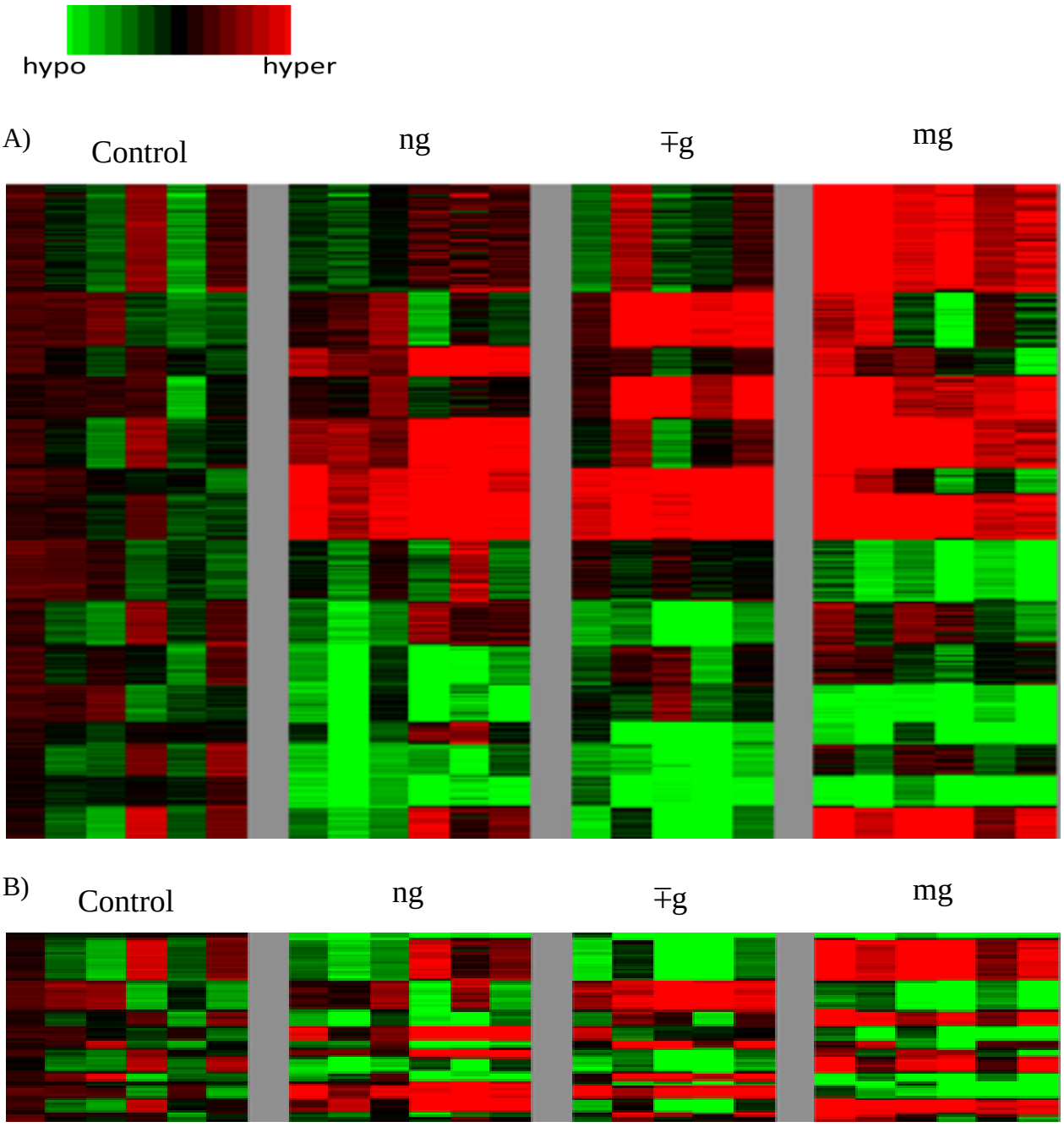
***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Table 4.12 Mediational regression analysis (Baseline Insulin). DNA methylation of *Irs-2*, *Jak-2*, and *TH* mediating baseline insulin (ng/ml) following perinatal BPA exposure in female offspring

Phenotype	Mediator	Exposure Group	Mean insulin (se)	Overall *P*-value	Mean insulin difference (se) vs control	*P*-value	Sample Size
Baseline Insulin	Full Sample Size (None)			0.13			
		mg	0.45 (.029)		-0.66 (0.42)	0.13	9
		ug	1.4 (0.27)		0.34 (0.4)	0.41	10
		ng	1.1 (0.29)		-.03 (0.42)	0.98	10
		control	1.1 (0.29)		-		10
	None			0.57			
	(model 1)	mg	0.78 (0.24)		-0.23 (0.35)	0.53	3
		ug	1.07 (0.24)		0.07 (0.35)	0.85	3
		ng	0.63 (0.24)		-0.38 (0.35)	0.31	3
		control	1 (0.24)		-		3
	Insulin Receptor Substrate-2			0.12			
	(model 4)	mg	-0.38 (0.54)		-2.08 (0.86)	0.05	3
		ug	1.19 (0.2)		-0.51 (0.38)	0.21	3
		ng	0.99 (0.25)		-0.71 (0.32)	0.06	3
		control	1.70 (0.36)				3
	Janus Kinase-2						
	(model 4)	mg	0.84 (0.22)		-0.41 (0.33)	0.25	3
		ug	1.15 (0.22)		-0.1 (0.32)	0.77	3
		ng	0.25 (0.31)		-1 (0.47)	0.07	3
		control	1.25 (0.26)		-		3
	Tyrosine Hydroxalase			0.53			
	(model 4)	mg	0.86 (0.27)		-0.14 (0.37)	0.70	3
		ug	1.07 (0.25)		0.06 (0.35)	0.87	3
		ng	0.55 (0.27)		-0.45 (0.37)	0.25	3
		control	1.0 (0.25)		-		3

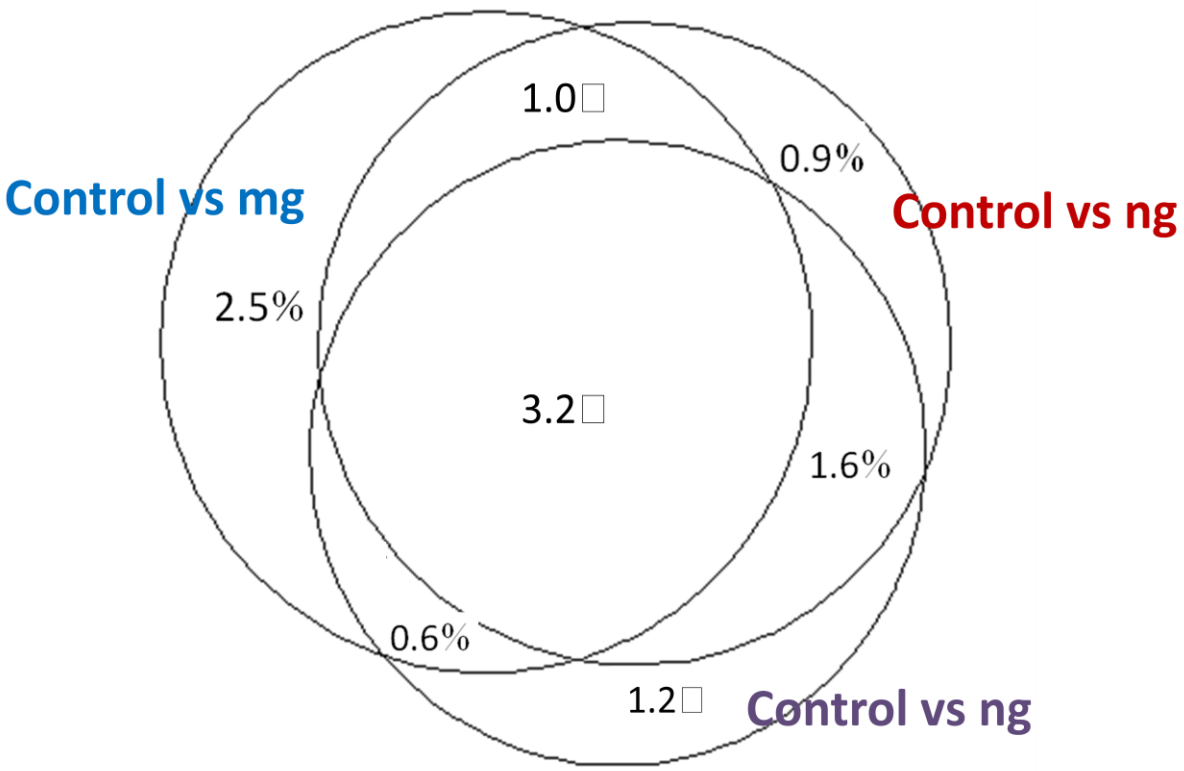
***represents >10% change in the difference of controls in model 1 compared to model 4 and a *P*-value of <0.10 in Models 1, 2, and 3.**

Figure 4.1 Heat map of top (hypermethylated) and bottom (hypomethylated) differentially methylated regions



A) Heat map indicating clusters of regions of hypo- or hypermethylation among 50 ng, 50 µg, and 50 mg BPA exposure groups compared to the controls. B) Heat map indicating clusters of regions of hypo- or hypermethylation of 50 ng, 50 µg, 50 mg BPA exposure groups and controls displaying non-monotonic effects across exposure groups.

Figure 4.2 Characterization of genome-wide methylation in BPA-exposed offspring



Venn diagram displaying the overlap of differentially methylated regions given the p-value cut-off of 0.001 among control, 50 ng, 50 µg, and 50 mg BPA exposure groups.

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CHAPTER 5

Conclusion

5.1 Overview

The body of evidence that poses BPA exposure as a human health threat continues to grow. Utilizing a murine model, the goal of this dissertation was to evaluate whether human physiologically relevant levels of BPA during development result in the disruption of metabolic homeostasis throughout the life-course, and whether these metabolic alterations are mediated by modifications to the epigenome. The aim of Chapter 2 was to evaluate gestational and lactational exposure to three physiologically relevant levels of BPA through the maternal diet on several metabolic phenotypes and hormones using sophisticated animal phenotyping in the offspring throughout their life-course. The aim of Chapter 3 was to determine whether the offspring exhibit modifications to the epigenome, in particular DNA methylation, after maternal dietary exposure to the three levels of BPA using a candidate gene and global methylation approaches in early life tissues (post-natal day 22 liver). The aim of Chapter 4 was to further determine whether the offspring exhibit modifications to DNA methylation, after perinatal exposure to the three levels of BPA using an epigenome-wide discovery approach in adult tissues (10 month liver). Furthermore, a mediational regression analysis was conducted to determine if the association of perinatal exposure to BPA and the metabolic phenotypes observed in the offspring in Chapter 2 were mediated by the alterations to DNA methylation discovered in adult liver tissue.

5.2 Conflicting Metabolic Data

The goal of Chapter 2 was to take a life-course approach in measuring metabolic and hormone phenotypes, incorporating sophisticated *in vivo* animal phenotyping in perinatally BPA exposed murine offspring. Human epidemiological studies examining BPA exposure and metabolic disorders are mostly of cross-sectional design, and thus are often limited to adult exposure at one time-point [Lang et al., 2008], and may be confounded by food consumption practices and/or body composition's effects on contaminant metabolism. Similarly, the majority of *in vivo* rodent studies examining the effects of pre- and/or neonatal BPA exposure on similar metabolic phenotypes only look at one time-point in the life-course. Although this is an adequate starting point in determining the effects of BPA on metabolic homeostasis, these approaches may not necessarily identify the full effects of early life BPA exposure on different life-stages [Ryan et al., 2010].

In a series of studies described herein, we determined that over the course of three time-points (3, 6, and 9 months of age) spanning young adulthood into mature adulthood, BPA exposed offspring exhibited hyperactive and lean phenotypes as well as improved hormonal parameters (9 and 10 months of age) with phenotypes being more prominent in females. Our observations of hyperactivity and lean body mass differ from the majority of epidemiological [Trasande L, 2012; Wang et al., 2012] and animal [vom Saal et al., 2012] studies that associate BPA with higher body weight and increased obesity, Varying results may be confounded by dietary practices, including diet fat and phytoestrogen composition, as well as altered BPA metabolism with body composition, especially in human cohorts. The finding of hyperactivity following perinatal BPA exposure is of significance as new Centers for Disease Control and Prevention (CD) data finds that attention deficit and hyperactivity disorder is seen in 11% of US

children as diagnoses rise [CDC, 2011]. The relevance of our work plays into the controversial debate of BPA acting as an environmental obesogen. This dissertation work shows evidence against BPA as an obesogen, and alternatively it reveals that BPA can influence other disease formation such as behavioral disorders.

Variability across rodent studies could be attributable to several factors that differ in experimental design. First, diet composition is not uniform across rodent models. The components making up each diet, such as the fat or protein content could play a role as a modifier to the effects of perinatal BPA exposure. Our study diet was phytoestrogen-free and on a non-high-fat diet background. Second, there are several routes of exposure to BPA such as dietary, *intraperitoneal* injection, and via subcutaneous osmotic pumps, for example. We opted to use a maternal dietary exposure as our administration route to best represent the major route of human BPA exposure during early development. Third, during development there are key developmental periods for different organ systems and varying exposure periods of BPA across studies may target different developmental stages. Our BPA exposure period, gestation and lactation, was set up to evaluate BPA's effect during epigenomic programming. Fourth, the species and strains of laboratory animals are inconsistent across experiments designed to evaluate the same outcomes. Genetic variability may influence the adverse outcomes, and thus, we need to take caution when translating health effects to human populations. Finally, metabolic phenotyping studies are lacking sophisticated animal phenotyping equipment and may be missing a comprehensive analysis of important metabolic measurements. We made use of an established animal phenotyping core set up with state-of-the art equipment allowing for periods of testing lasting for several days (Michigan Nutrition Obesity Research Center, Animal Phenotyping Core, Ann Arbor, MI).

The range of experimental designs in studies with a common outcome, in this case metabolic homeostasis, makes the interpretation of the collective results difficult. With varying outcomes, we must take caution when deciphering whether BPA is a threat to human health, and take into consideration the variability in experimental design across the animal literature as described above. BPA exposure during several key time-points in development may result in various disease outcomes later in life, making it crucial to measure the biological pathways involved in BPA exposure which will enable researchers to develop therapeutic or preventative strategies for varying disease states after exposure to environmental insults.

5.3 Epigenetics as Target Mechanism

Epigenetic fetal programming via DNA methylation may provide a pathway for BPA exposure to influence disease susceptibility [Jirtle et al., 2007]. The goals of chapters 3 and 4 were to determine if BPA exposure during early development resulted in modifications to the epigenome, specifically to DNA methylation. We examined these modifications by using two approaches including a candidate gene driven, as well as an epigenome-wide discovery platform. Both of these approaches illustrated that BPA exposure via the maternal diet is influential on offspring DNA methylation programming during early development.

5.3.1 Candidate Gene and Global DNA Methylation

Several candidate genes including two metastable epialleles, two imprinted genes, and three genes chosen from the literature based on our original hypothesis that BPA would result in obesity related phenotypes were examined. In addition, global methylation at CCGG sites throughout the genome were measured. Non-monotonic effects of BPA on the epigenome were

often observed. First, the higher dietary exposure to BPA led to hypomethylation at the A^{vy} metastable epiallele, while lower dietary levels were less effective at hypomethylating. Second, the middle BPA exposure dose resulted in hypermethylation at the $Cabp^{IAP}$ metastable epiallele, while the low and high BPA exposures did not result in altered methylation levels. Third, at the imprinted locus *Igf2*, the low and high BPA exposure doses resulted in hypermethylation while the middle dose did not have altered methylation. Lastly, at the imprinted locus *H19*, the middle BPA exposure dose led to hypomethylation while the low and high doses did not result in altered methylation levels. These data are significant because they (1) provide mechanistic evidence for previously observed non-monotonic dose-dependent effects of BPA, and (2) provide evidence that variable dose levels of BPA may act across different biological pathways. Global methylation of CCGG sites resulted in increased methylation across all BPA doses. There were no significant changes in DNA methylation at three of the genes (*HsdBeta 11 Type2, Ppar-alpha, Ppar-gamma*) chosen from the literature.

The majority of studies focused on epigenetic alterations following developmental environmental insults are focused on candidate gene approaches. This approach is useful if you are interested in a specific region of the genome or a very specific disease trait. It is of importance to note that the mouse genome contains over 23,000 genes. Thus, when utilizing a candidate gene approach, it is strictly limited by its reliance on the priori knowledge about the physiological, biochemical, or functional aspects of all possible candidates. While the identification of epigenetic processes of candidate genes affected by endocrine disrupting hormones such as BPA has its limitations, significant progress in modern technologies allowing for unbiased epigenome-wide investigation across tissues and species has been developed over the past decade.

5.3.2 Epigenome-wide Discovery

The use of epigenome-wide discovery tools combined with biological pathway analysis to identify the constellation of genes undergoing BPA dose-dependent epigenetic dysregulation in disease pathways combined with a focused candidate gene validation also serves as a comprehensive approach to identifying the underlying mechanisms of BPA associated metabolic sequela. We used an epigenome-wide platform that included about 720,000 probes in over 20,000 mouse promoter regions to characterize regions of altered methylation, followed by focused validation of altered methylation at candidate loci. About 380,000 probes exhibited differentially methylated regions by BPA exposure in at least one of our three comparisons (ng versus control; ug versus control; and mg versus control).

The promoter epigenome-wide platform that we utilized worked as a tool in discovering novel epigenetic biomarkers of perinatal BPA exposure and its role in metabolic homeostasis. This epigenomic tool serves as a "semi-unbiased" approach because it is limited to CpG islands located in promoter regions. It is important to note that epigenetic modifications to DNA methylation outside of promoter regions have recently been discovered to play a role in gene expression [Wang, 2012]. Future work should expand to other epigenome-wide technology that covers all other epigenomic regions of potential interest.

5.4 Role in the Field of Public Health

BPA is an environmental exposure of concern because it is ubiquitous in the environment and is found in the majority of human populations that it is tested in. Animal models of early life BPA exposure have indicated that it poses a threat to human health. Studies are continually

exploring BPA exposure and health outcomes in order to discover the possible role that it has on different disease states. However, there are limited studies that explore the possible mechanisms that BPA is acting upon, and additionally, linking the mechanism to the disease state. The aim of this dissertation was to investigate the role of perinatal BPA exposure on metabolic homeostasis and DNA methylation. Lastly, DNA methylation as a mediating factor on the association of BPA exposure and metabolic phenotypes was further tested in order to enable clinical and public health practices to apply and develop epigenetically-driven therapeutic and preventative interventions. Two top hit candidate genes were found to play a role as possible mediators in the development altered hormone levels after exposure to perinatal BPA.

The elucidation of modifications to epigenomic loci associated with metabolic dysregulation following human physiologically relevant levels of BPA will strengthen health risk assessment and shape diagnostic and therapeutic strategies for disease states such as obesity and other metabolic disorders. The identification of epigenetic exposure biomarkers critical in metabolic regulation will be useful in the recognition of at-risk individuals prior to disease onset. Also, unlike genetic mutations, epigenetic profiles are potentially reversible; therefore, epigenetic approaches for prevention and treatment, such as nutritional supplementation, may be developed to counteract negative epigenomic profiles. Additionally, investigating the interactions among micronutrients required in one-carbon metabolism and those that may indirectly affect their supply to maintain cycle efficiency can be implicit by integrating a genome-wide application. If we determine that BPA exposure results in altered methylation of epigenetic loci important for metabolic homeostasis or negative changes in obesity-related physiological and hormonal assessments, it would be important to determine if nutritional

supplements (e.g. folic acid and genistein) not only during development, but also later in life can protect against these negative effects.

5.5 Future Work

Although there is a continual concern of the health effects following BPA exposure, an increasing number of products are replacing BPA with compounds such as bisphenol F (BPF) and bisphenol S (BPS) [Liao et al., 2013]. Evidence already reveals that these BPA replacements interfere with estradiol-mediated pathways [Viñas, 2013]. Additionally, other plasticizers such as phthalates, that are also ubiquitous throughout the environment, have been associated with obesity [Meeker, 2012]. For instance, a recent *in vitro* experiment showed that phthalates work as a *PPAR-gamma* agonist resulting in accelerated adipogenesis [Taxvig et al., 2012]. Furthermore, increasing levels of urinary phthalate metabolites were positively associated with adiposity measured via waist circumference and decreased beta cell function in male NHANES (1999-2002) participants [Stahlhut et al., 2007]. The perpetual presence of endocrine disrupting chemicals in the environment presents the opportunity for further study designs such as determining the safety of replacement bisphenols (BPF and BPS, for example) as well as evaluating the interaction of several endocrine disrupting chemicals (EDC)s on human health given the notion that individuals are exposed to multiple EDCs at any given time [Talsness et al., 2009].

Because the epigenome is particularly sensitive at various time-points throughout the life-course, targeted population interventions may be efficient and cost-effective. Continued animal epigenomic models translated into human research will strengthen our understanding in the biological pathways associated with environmental exposure, diet, and human health. Therefore,

the combination of nutrition and epigenomic research holds tremendous potential not only for individualized health, but also for diagnostic, screening, and prevention strategies for population-wide disease.

5.7 References

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